Study of Actinobacteria and their Secondary Metabolites from Various Habitats in Indonesia and Deep-Sea of the North Atlantic Ocean

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List of Abbreviations

°C	Degree Celsius
16S rRNA	Component of the 30S small subunit of a prokaryotic ribosomal ribonucleic acid
[M+H]+	Protonated molecular ion
ASW	Artificial sea water
DAD	Diode array detector
DAP	2, 6-Diaminopimelic acid
DDH	DNA-DNA hybridization
DNA	Deoxyribonucleic acid
DNP	Dictionary of natural products
EDTA	Ethylenediaminetetraacetic acid
G+C	Guanine and cytosine
HCV	Hepatitis C virus
HEPES	(4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HR-ESI-MS	High resolution electron spray ionization mass spectrometry
ISP	International *Streptomyces* project
JSRM	Jump start ready mix
LC-MS	Liquid chromatography – mass spectrometry
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MIC	Minimal inhibition concentration
MLSA	Multilocus sequence analysis
NMR	Nuclear magnetic resonance
NRPS	Nonribosomal peptide synthetases
OD	Optical density
OSMAC	One strain-many compounds
PCR	Polymerase chain reaction
PKS	Polyketide synthases
RNA	Ribonucleic acid
rpm	Revolutions per minute

Abstract

Actinobacteria are Gram-positive bacteria and the prolific producers of bioactive molecules. In the course of drug discovery from actinobacterial sources, some samples were taken from different locations and habitats of Indonesia and isolation of Actinobacteria was conducted thereof. Totally 196 strains of Actinobacteria were isolated, and approximately half of them were characterized by 16S rRNA gene analysis. Around two-thirds of them were detected as *Streptomyces*. One of the *Streptomyces* strains, i.e., strain SHP 1-2 produced active extracts against some microbial pathogens. Analysis and isolation of compounds from its extract revealed that two novel molecules, which are indolactam derivatives, were found from it. Polyphasic taxonomy study of strain SHP 1-2 suggested that this strain is a novel species in the *Streptomyces* group. Another study from different strains suggested that strain MAE-11, isolated from mangrove area, was identified close to *Kitasatopora* and *Streptomyces* species and generated active extract against hepatitis C virus (HCV). The extract was known to contain bafilomycin D as the molecule that was responsible for the antiviral activity. Isolate 196526CR was detected as *Amycolatopsis* species and could produce an extract that showed activity against some tested microbes. The compound analysis and isolation from its extract indicated that nitrosoxacin C possesses property as an antimicrobial compound. Chemotaxonomy study of strain ASO4wet, which was previously isolated from deep-sea in the North Atlantic Ocean, was also conducted and the data supported that ASO4wet belongs to the genus *Streptomyces*. The strain is described as the novel species.

Keywords: Actinobacteria, *Streptomyces*, drug discovery, Indonesia, deep sea

Abstrakt

Actinobakterien sind Gram-positive Bakterien und die ergiebigsten Produzenten bioaktiver Moleküle. Im Zuge der Wirkstoffforschung aus Actinobakterien wurden einige Proben an verschiedenen Orten und Lebensräumen Indonesiens entnommen und daraus Actinobakterien isoliert. Insgesamt wurden 196 Aktinobakterien-Stämme isoliert und etwa die Hälfte von ihnen wurde durch 16S-rRNA-Genanalyse charakterisiert. Etwa zwei Drittel von ihnen wurden als *Streptomyces* identifiziert. Einer der *Streptomyces*-Stämme, d. h. Stamm SHP 1-2, produzierte aktive Extrakte gegen einige pathogene Mikroorganismen. Nach Analyse und anschließender Isolierung der Inhaltsstoffe konnten zwei neue Substanzen, die zu der Indolactam-Derivaten gehören, gefunden werden. Eine polyphasische Taxonomiestudie des Stammes SHP 1-2 legte nahe, dass dieser Stamm zu einer neuen Spezies in der Gattung *Streptomyces*-gehört. Eine vergleichende Studie mit verschiedenen Stämmen legte nahe, dass der Stamm MAE-11, der aus Mangrovengebieten isoliert wurde, nahe verwandt mit *Kitasatopora*- und *Streptomyces*-Arten ist und einen aktiven Extrakt gegen das Hepatitis C-Virus (HCV) produziert. Es zeigte sich, dass der Extrakt Bafilomycin D als die Substanz enthielt, die für die antivirale Aktivität verantwortlich war. Das Isolat 196526CR wurde als *Amycolatopsis*-Spezies identifiziert und bildet einen Extrakt, der eine Aktivität gegen einige Mikroorganismen zeigte. Die Analyse der Bestandteile und die Isolierung aus diesem Extrakt zeigten, dass Nitrosoxacin C für die antimikrobielle Eigenschafte verantwortlich ist. Eine chemotaxonomiche Studie des Stammes ASO4wet, der aus der Tiefsee im Nordatlantik isoliert worden war, wurde ebenfalls durchgeführt, und die Daten untermauerten, dass ASO4wet zur Gattung *Streptomyces* gehört. Der Stamm wird als neue Art beschrieben.

Schlüsselwörter: Aktinobakterien, *Streptomyces*, Wirkstoffforschung, Indonesien, Tiefsee

1 Introduction

1.1 Natural product and drug discovery

Natural products are various chemical compounds produced by bacteria, fungi, plants, and animals. These include the primary metabolites such as DNA, RNA, and protein and the secondary metabolites such as isoprenoids, alkaloids, polyketides, and peptides, especially nonribosomal peptides[1–4]. However, the definition of natural product usually is used by many scientists to refer to the secondary metabolites produced by organisms[1]. Not like the primary metabolites, which are very important for the growth and reproduction, the secondary metabolites give other bioactivity benefits to the producing organisms. They could be used for defensive or attack mechanisms, competition with the other organisms, and interspecies communication. Therefore, it can be considered that the secondary metabolites are required for survival of the hosts in their environment[5].

More than one million of natural products have been isolated recently. They are essential and valuable agents in human life. People have used them for many applications such as in nutrition, medicine and agriculture. Many of them originate from plants (50-60%) such as alkaloids, flavonoids, terpenoids, and steroids and microbes produce only a small portion (about 5%). However, only 20-25% of these compounds possess biological activity and circa 10% of these active molecules are produced by microbes, especially from Actinobacteria which contribute around 45% followed by fungi (~38%)[6–8] as shown in Figure 1.

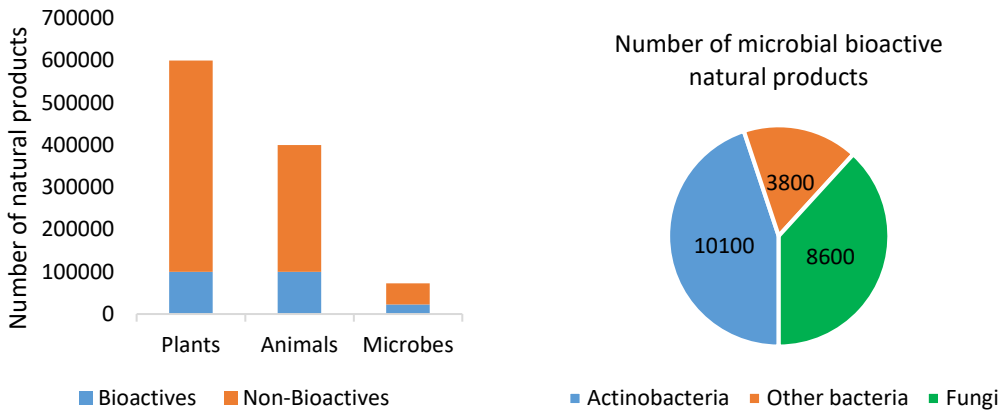


Figure 1. Distribution of natural products produced by organisms. Data are derived from Bérdy 2005[8].

1 Introduction

Microbes have supplied compounds they produced that have helped people to overcome diseases for 70 years. These comprise antibiotics, antitumor drugs, immunosuppressants, enzyme inhibitors, and antiviral agents[2]. The history of drug discovery from microbial sources has been started since 1929 when Alexander Fleming published his work about the antimicrobial activity from the mould *Penicillium notatum* that killed *Staphylococcus aureus*[9]. Since then, many scientists have worked to find drugs from microbial sources. In 1942, Waksman and Tishler published their finding of actinomycin which was produced by *Actinomyces antibioticus* (the current name is *Streptomyces antibioticus*) that has antibacterial activity[10].

The "heroic" or "golden era" of antibiotics is between the 1940s-1950s when almost all valuable antibacterial drugs were found, such as tetracyclines, cephalosporins, aminoglycosides, and macrolides. It was suggested that in the period between the 1950s-1960s, the major issues in chemotherapy had been resolved. Interestingly, antibiotics discovered in this era were predominantly produced by *Streptomyces* species. *Streptomyces* was reported to produce 70-80% of the all discovered antibiotics, which are mainly active as antibacterial and antifungal agents. However, there were some false classification of *Steptomyces* species, e.g., *Streptomyces erythraeus*, which was then amended to *Saccharopolyspora erythraea*. In this period, also, the discovery of other bioactivities such as antitumor, antiviral, and enzyme inhibitor had just commenced[8,11].

In the 1970s-1990s, because of the cost of research had raised, the research had become laboriously. The novel compounds that were discovered in this period were primarily analogues of previously known metabolites. The area of research had become broad such as finding molecules for antitumor and agricultural antibiotics. In this period, problems of the emerging new pathogens and the growing of multi-resistant strains had become essential issues to be resolved. In the same time, it was also reported that many novel compounds produced from "rare actinomycetes" had been isolated and elucidated. This "rare actinomycetes" include the genera from actinomycetes beside *Streptomyces* such as *Micromonospora*, *Actinomadura*, *Streptoverticillium*, *Actinoplanes*, *Nocardia*, *Saccharopolyspora* and *Streptosporangium*[8].

From the 1990s until nowadays, the reports of the novel isolated compounds have been dramatically increased; however, these mostly belong to non-antibiotic molecules, analogous, and minor metabolites. The amount of novel chemical types had decreased. It is also recognized that there was an innovation gap in the period between 1962 and 2000, that means there were no primary classes of antibiotics introduced (Figure 2)[8,12].

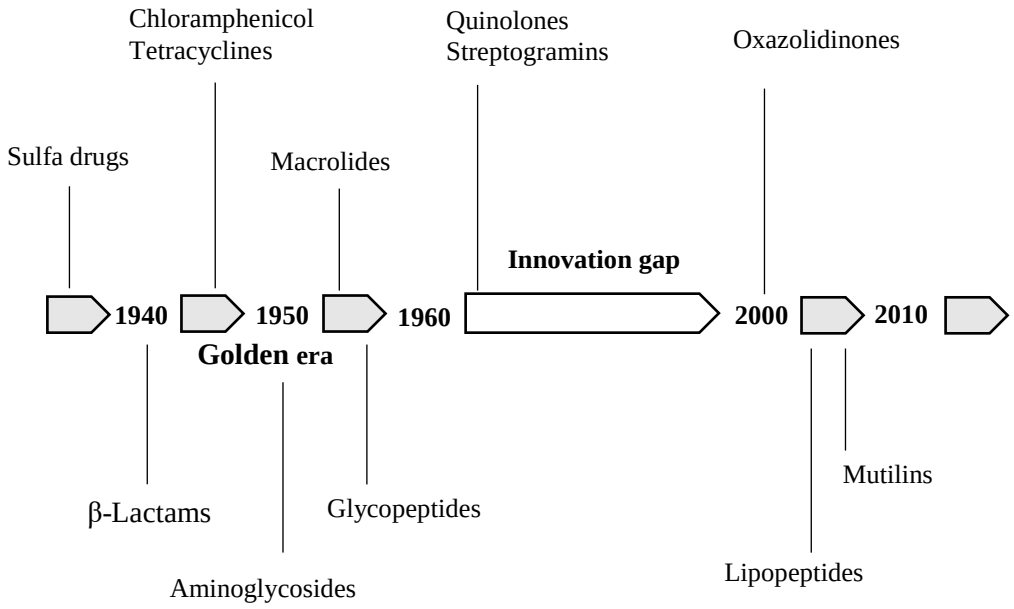


Figure 2. Timeline of introduced antibiotics. Adapted from Fischbach and Walsh[13].

On the other hand, the problems of multi-resistant strains, emerging new pathogens and re-emerging pathogens are increasing recently. The issues should be resolved; therefore, novel strategies are needed for discovering novel drugs. Some novel approaches for drug discovery have been carried out in recent times. These include the biodiversity-based method, modifying cultivation conditions, and genome mining[8,14].

In the biodiversity based method, finding the novel species of bacteria is required in the effort of finding new antibiotics. There is a close relationship, based on the investigation during recent decades, between the discovering of new antibiotics and the description of novel bacteria species particularly within the Actinobacteria and gliding bacteria. It is suggested that the possibility of finding out novel compounds with unique structures are undoubtedly associated with the novel species. The method for isolation the novel species can be carried out by exploring the unexplored habitats such as desert, deep sea, and endosymbiotic environment. One of the underexplored countries, for Actinobacteria biodiversity, is Indonesia. Indonesia has an enormous territory encompassing 17,000 islands with diverse habitats and as one of the countries

having vast biodiversity in the world. Therefore, isolating Actinobacteria from Indonesian samples may increase the chance of either finding novel species or bioactive compounds. The other method which is still related to the biodiversity is by isolating and studying the bacteria which are not part of the taxa of eminent antibiotic producers[14–16].

Some previous investigations suggested that the secondary metabolite profile of a bacterial species is possibly changed by modifying the culture conditions. By using whole-genome sequencing, it is finally understood that most fungi and bacteria have the capacity to generate more compounds than they usually produce in the standard cultivation condition. Changing the cultivation condition may be carried out by altering some parameters such as the concentration of phosphate in the medium, media composition, aeration, culture container, and addition of some enzyme inhibitors, solvents, and heavy metals. The other method is by using the co-culture method either with live or dead cells. By conducting such modifications, the probability to enhance the number of compounds produced by one strain is postulated become high. This method is known as OSMAC (One Strain-Many Compounds). The technique is suggested to stimulate the expression of the silent (cryptic) metabolic pathways in the microbial strains that can improve the variety of compounds they generate. However, this method is a random technique that is difficult to establish general standards for all strains[14,17–19].

Genome mining is the method of retrieving information from genome sequences of species. This method can be conducted by detecting and analyzing the biosynthetic gene clusters of secondary metabolites and afterwards associating it to the corresponding chemical entities. Biosynthetic gene clusters are the main structures of the biosynthetic pathway of compounds at the genome level, which typically encode multidomain enzymes, such as polyketide synthases (PKS), nonribosomal peptide synthetases (NRPS), transporters, and some tailoring enzymes. The tailoring enzymes are some enzymes needed for a specific reaction such as halogenation, oxidation, glycosylation, and cyclisation. The determination of the biosynthetic gene cluster can be accomplished base on homology with notorious secondary metabolite gene clusters. This homology is obtained from the classification of previously recognized biosynthetic gene clusters based on the biosynthetic pathway, domain structure, conserved motifs, hidden Markov models, and chemical structure groups of encoded

compounds. The information derived from biosynthetic gene clusters can be employed to different methods such as guiding a more targeted drug discovery technique, peptide and glycogenomic approaches, and enabling the heterologous expression in the best expression host. However, it is still challenging to associate biosynthetic gene clusters to bioactivity[14,15,20,21].

1.2 Actinobacteria

The phylum Actinobacteria is one of the most prominent taxonomic and essential groups among the primary lineages within the Gram-positive bacteria group. They are mostly characterized by having high guanine and cytosine (G+C) content in their genomes Actinobacteria comprises soil dwellers (*Streptomyces* spp.), plant symbionts (*Leifsonia* spp.), nitrogen-fixing commensals (*Frankia*), and gastrointestinal tract inhabitants (*Bifidobacterium* spp.). However, small numbers of Actinobacteria are recognized as pathogens such as *Mycobacterium* spp. (e.g. *Mycobacterium tuberculosis* that causes tuberculosis (TB) in humans), *Nocardia* spp., *Tropheryma* spp., *Corynebacterium* spp., and *Propionibacterium* spp. Most of Actinobacteria are aerobic, heterotrophic, and can be found in both terrestrial and aquatic ecosystems (including marine habitats)[22,23].

They generate about two-thirds of all clinically used antibiotics from natural products. Many Actinobacteria produce a mycelium like fungi. Because of this, they are considered as the transitional organisms between fungi and bacteria. The mycelium formed Actinobacteria reproduce by sporulation and are called actinomycetes, which originated from the Greek words for ray (aktis or aktin) and fungi (mukēs). The difference between actinomycetes and fungi is that the former have no nucleus, contain peptidoglycan in their cell wall, and are susceptible to antibacterial agents. Most of them are saprophytic and soil-dwelling bacteria that spend most of their life cycles as semi-dormant spores, particularly in the condition when their nutrients are scarce. They mainly grow better at a pH 6-9, with the most optimal growth around neutrality and are mostly mesophilic, which means that the temperature for their optimal growth is between 25-30°C[22].

The phylum Actinobacteria has six classes. These include Acidimicrobiia, Actinobacteria, Coriobacteriia, Nitriliruptoria, and Thermoleophilia. The class Actinobacteria encompasses 29 orders and 62 families, as shown in Table 1. From 62 families in the phylum Actinobacteria, around 74% of them belong to the class

Actinobacteria (https://www.ncbi.nlm.nih.gov/taxonomy). Delineation of species in Actinobacteria is conducted by phenotypic and genotypic analysis. The primary phenotypic characteristics employed to delineate the taxonomy of Actinobacteria are based on the microscopic morphology and chemotaxonomy. For genotypic analysis, the taxonomic study of Actinobacteria can be carried out based on the 16S rRNA gene analysis, DNA-DNA hybridization, multilocus sequence analysis (MLSA), and genome sequencing[22,24].

Table 1. Current taxonomic outline for the phylum Actinobacteria. The data are based from taxonomy database in NCBI website (https://www.ncbi.nlm.nih.gov/taxonomy)

Phylum	Class	Order	Family	Genus
Actinobacteria	Acidimicrobiia,	Acidimicrobiales	4 Families	10 genera
	Actinobacteria,	Acidothermales	1 Family	1 genera
		Actinomycetales	1 Family	11 genera
		Actinopolysporales	1 Family	2 genera
		Bifidobacteriales	1 Family	10 genera
		Catenulisporales	2 Families	3 genera
		Corynebacteriales	7 Families	20 genera
		Cryptosporangiales	1 Family	2 genera
		Frankiales	2 Families	4 genera
		Geodermatophilales	1 Family	5 genera
		Glycomycetales	1 Family	5 genera
		Jiangellales	1 Family	3 genera
		Kineosporiales	1 Family	6 genera
		Micrococcales	16 Families	161 genera
		Micromonosporales	1 Family	33 genera
		Nakamurellales	1 Family	1 genera
		Propionibacteriales	2 Families	33 genera
		Pseudonocardiales	1 Family	35 genera
		Sporichthyales	1 Family	3 genera
		Streptomycetales	1 Family	6 genera
		Streptosporangiales	3 Families	35 genera
	Coriobacteriia,	Coriobacteriales	2 Families	10 genera
		Eggerthellales	1 Family	15 genera
	Nitriliruptoria,	Egibacterales	1 Family	1 genera
		Egicoccales	1 Family	1 genera
		Euzebyales	1 Family	1 genera
		Nitriliruptorales	1 Family	1 genera
	Thermoleophilia	Solirubrobacterales	4 Families	4 genera
		Thermoleophilales	1 Family	1 genera

1.2.1 Streptomyces

Bacteria of the genus *Streptomyces* can produce a secondary metabolite named geosmin. This compound does not have any antibiotic activity; however, it gives the soil its distinctive smell. They are perfectly adapted to survive in the soil where they grow by forming substrate mycelium that can help them to get nutrients. They secrete various enzymes that can digest insoluble organic polymers and numerous secondary metabolites that may be suggested not only as chemical weapons for killing other soil organisms but also as signalling molecules for modulating the metabolic process in target organisms. They also have antibiotic resistance genes to protect themselves from their own produced antibiotics. These resistance genes, however, can be transferred to other bacteria via horizontal gene transfer. The life cycle of *Streptomyces* starts with spore germination, forming vegetative hyphae, and continues to create substrate mycelium. The exponential growth of the vegetative hyphae is accomplished through a combination of tip extension and branching. The cell division during vegetative growth creates cross-walls that separate the hyphae into connected compartments. In response to unfavourable conditions such as nutritional deficiency and other stress signals, the vegetative mycelium differentiates to form reproductive aerial hyphae, which undergo cellular division to produce spores. Most of the antibiotics are formed in this differentiation moment; however, many industrial processes for secondary metabolite production from *Streptomyces* are accomplished with liquid cultures. Under these conditions, *Streptomyces* strains usually do not sporulate[22,25,26]. The morphology of *Streptomyces* species can be seen in Figure 3.

Streptomyces strains can produce various antibiotics with different structures and functions such as inhibition of DNA replication and the synthesis of RNA, cell wall, and protein. Some examples of antibiotics produced by *Streptomyces* species are streptomycin from *S. griceus*, cephalosporin from *S. clavuligerus*, chloramphenicol from *S. venezuelae*, daptomycin from *S. roseosporus*, and novobiocin from *S. niveus*. Streptomycin and chloramphenicol are the inhibitors of bacterial protein synthesis. Streptomycin binds to the small 16S rRNA of the 30S ribosomal subunit, while chloramphenicol binds to 23S rRNA of the 50S ribosomal subunit. Cephalosporin was firstly isolated from fungus *Acremonium chrysogenum* and then it was known that *Streptomyces* also can produce the compound. It disrupt the synthesis of the bacterial

cell wall, while daptomycin can break up the cell membrane. As for novobiocin, it has another mechanism of antibacterial activity by binding to DNA gyrase[27,28]. Some other antibiotics produced by *Streptomyces* species are shown in Table 2.

Figure 3. *Streptomyces* sp. strain C196921 in agar medium

Table 2. Some antibiotics produced by *Streptomyces* species

Antibiotic	Class of molecule	Function	Producer	Reference
Arylomycin	Lipopeptides	Inhibition of type I signal peptidase	*Streptomyces* sp. Tu 6075	[29,30]
Capreomycin	Peptides	Inhibition of protein synthesis	*S. vinaceus* *S. capreolus*	[31]
Cephalosporins	β-Lactams	Inhibition of cell wall synthesis	*S. clavuligerus*	[32,33]
Chloramphenicol	Chloramphenicols	Inhibition of protein synthesis	*S. venezuelae*	[34,35]
Cycloserine	Analog of D-alanine	Inhibition of cell wall synthesis	*S. garyphalus*	[36,37]
Daptomycin	Lipopeptides	Destruction of the membrane potential	*S. roseosporus*	[38]

Fosfomycin	Fosfomycin	Inhibition of cell wall synthesis	*S. fradiae*	[39]
Kanamycin	Aminoglycosides	Inhibition of protein synthesis	*S. kanamyceticus*	[40]
Lincomycin	Lincosamides	Inhibition of protein synthesis	*S. lincolnensis*	[41]
Neomycin	Aminoglycosides	Inhibition of protein synthesis	*S. fradiae*	[42,43]
Novobiocin	Aminocoumarins	Inhibition of DNA gyrase	*S. niveus*	[44,45]
Oleandomycin	Macrolides	Inhibition of protein synthesis	*S. antibioticus*	[46,47]
Platensimycin	Platensimycin	Inhibition of fatty acid production	*S. platensis*	[48]
Pristinamycin	Streptogramins	Inhibition of protein synthesis	*S. pristinaespiralis*	[49]
Ribostamycin	Aminoglycosides	Inhibition of protein synthesis	*S. ribosidificus*	[50]
Spiramycin	Macrolides	Inhibition of protein synthesis	*S. ambofaciens*	[51]
Streptomycin	Aminoglycosides	Inhibition of protein synthesis	*S. griseus*	[52]
Tetracycline	Tetracyclines	Inhibition of protein synthesis	*S. aureofaciens*	[53,54]
Viomycin	Peptides	Inhibition of protein synthesis	*S. vinaceus*, *S. capreolus*	[31]
Virginiamycin	Streptogramins	Inhibition of protein synthesis	*S. virginiae*	[55]

1.2.2 Non-*Streptomyces* Actinobacteria

In contrast to the member of the genus *Streptomyces* that produce around 74% of antibiotics from Actinobacteria sources, non-*Streptomyces* Actinobacteria contribute

only about 26% of the antibiotics of their origin. This non-*Streptomyces* Actinobacteria are called rare actinomycetes, which comprise more than 200 genera e.g., *Actinomadura*, *Actinoplanes*, *Amycolatopsis*, *Actinokineospora*, *Acrocarpospora*, *Actinosynnema*, *Catenuloplanes*, *Cryptosporangium*, *Dactylosporangium*, *Kibdelosporangium*, *Kineosporia*, *Kutzneria*, *Microbiospora*, *Micromonospora*, *Microtetraspora*, *Nocardia*, *Nonomuraea*, *Planomonospora*, *Planobispora*, *Pseudonocardia*, *Saccharomonospora*, *Saccharopolyspora*, *Saccharothrix*, *Streptosporangium*, *Streptoverticillium*, *Spirilliplanes*, *Thermomonospora*, *Thermobifida*, and *Virgosporangium*[8,56]. The morphology of some non-*Streptomyces* can be seen in Figure 4.

Rare actinomycetes are frequently referred to the strains of Actinobacteria whose isolation rate is considerably less than that of the *Streptomyces* strains isolated by classical approaches. In comparison with *Streptomyces*, they have not been extensively investigated in previous years and might become new sources of novel secondary metabolites. These bacteria are extensively distributed both in terrestrial and aquatic ecosystems. Their distribution is influenced by some environmental elements such as soil type, pH, humus content, and the characteristics of the humic acid content of the soil. While non-*Streptomyces* Actinobacteria may increase the probability of discovering novel compounds, their genetics and physiology are not well understood[56].

Amycolatopsis sp. strain 196526CR | *Pseudonocardia* sp. strain 5931 | *Microbispora* sp. strain 190401

Figure 4. Some non-*Streptomyces* strains in agar medium

The prolific group of non-*Streptomyces* Actinobacteria is represented by *Actinoplanes* strains, which have significantly been isolated by utilizing the properties of their

spores that are mobile and have chemotactic movement. These groups of bacteria produce more than 120 antibiotics such as teicoplanin, ramoplanin, purpuromycin, lipiarmycin, and actagardine. Another group of non-*Streptomyces* Actinobacteria isolated on an enormous scale over the last few years belong to the genus *Micromonospora*. This genus is regarded as the second biggest group of culturable Actinobacteria in soil and can be isolated by using selection medium containing antibiotics such as gentamicin and novobiocin or with the pre-treatment by using toxic agents such as phenol and chlorhexidine gluconate solutions because their spores are resistant to these chemicals. *Micromonospora* strains produce some important antibiotics such as gentamicin, sisomicin, fortimicin, mycinamicins, rosamicins, and everninomycin[57]. Some other antibiotics produced by non-*Streptomyces* species are listed in Table 3.

1.3 Polyphasic taxonomy of the class Actinobacteria

Taxonomy, which is the synonym of systematics or biosystematics, is essential in studying organisms. It comprises three main parts, i.e., classification, nomenclature, and identification. Classification can be carried out by organizing organisms based on similarity into taxonomic groups. The term nomenclature includes giving the label of the units determined in classification. Identification means the process of deciding whether an organism is a member of one of the units determined in classification and labelled in nomenclature. Taxonomic information helps scientists to figure out the biodiversity and correlation among organisms from distinct ecological systems. Taxonomy in prokaryotes has an indispensable function in supporting the accurate identification of microbial strains from difference varieties[58,59].

Table 3. Some antibiotics produced by non-*Streptomyces* species

Antibiotic	Class of molecule	Function	Producer	Reference
Abyssomycin	Spirotetronates	Inhibition of folate biosynthesis	*Verrucosispora* sp. AB-18-032	[60]
Actagardine	Polycyclic peptides	Inhibition of cell wall synthesis	*Actinoplanes garbadinensis*	[61]
Borrelidin	Macrolides	Inhibition of protein synthesis	*Nocardiopsis* sp. HYJ128	[62,63]

Branimycin	Macrolides	Inhibition of DNA synthesis	*Pseudonocardia carboxydivorans* M-227	[64,65]
Cationomycin	Polyethers	Modification of cell membrane	*Actinomadura azurea*	[66]
Everninomycin	Oligosaccharides	Inhibition of protein synthesis	*Micromonospora carbonacea*	[67,68]
Fortimicin	Aminoglycosides	Inhibition of protein synthesis	*Micromonospora* olivoastrospora	[69,70]
Gentamicin	Aminoglycosides	Inhibition of protein synthesis	*Micromonospora purpurea*	[71]
Kibdelomycin	Kibdelomycin	Inhibition of DNA synthesis	*Kibdelosporangium* sp. MA7385	[72]
Kijimicin	Polyethers	Modification of cell membrane	*Actinomadura* sp. MI215- NF3	[73]
Lipiarmycin	Macrolides	Inhibition of RNA synthesis	*Actinoplanes deccanensis*	[74,75]
Madurahydroxylactone	Benzo[a]naphtha-cenequinones	Inhibition of cell-division protein FtsZ	*Nonomuria rubra*	[76,77]
Mycinamicin	Macrolides	Inhibition of protein synthesis	*Micromonospora griseorubida*	[78,79]
Nargenicin	Macrolides	Inhibition of DNA synthesis	*Nocardia devorans*, *Nocardia* sp. CS682	[80,81]
Purpuromycin	Naphthoquinones	Inhibition of protein synthesis	*Actinoplanes ianthinogenes*	[82,83]
Ramoplanin	Lipodepsipeptides	Inhibition of cell wall synthesis	*Actinoplanes ramoplaninifer*	[84,85]
Rifamycin	Ansamycins	Inhibition of RNA synthesis	*Amycolatopsis mediterranei*	[86]
Rosamicin	Macrolides	Inhibition of protein synthesis	*Micromonospora rosaria*	[87,88]
Saccharomicin	Oligosaccharides	Disruption of cell membrane	*Saccharothrix espanaensis*	[89]

Sisomicin	Aminoglycosides	Inhibition of protein synthesis	*Micromonospora* inyoensis	[90,91]
Teicoplanin	Glycopeptides	Inhibition of cell wall synthesis	*Actinoplanes teichomyceticus*	[92]
Vancomycin	Glycopeptides	Inhibition of cell wall synthesis	*Amycolatopsis orientalis*	[93]

Bacterial taxonomy commenced in the late 19th century when the classification was only based on simple phenotypic markers such as morphology, growth requirements or pathogenicity. Subsequently, the other properties, i.e., physiological and biochemical, were added for this purpose. In the period of 1960s-1980s, chemotaxonomy, numerical taxonomy and DNA–DNA hybridization techniques were applied in studying taxonomy. Later, in the 1980s, the emergence of DNA amplification and sequencing techniques, especially of the 16S rRNA gene, created significant progress in bacterial classification. Since the mid-1990s, whole-genome sequencing cretaed an innovation by providing an approach to obtain the complete genetic information of a single strain[59].

In order to establish reliable taxonomy of bacteria, integration of phenotypic, genotypic, and phylogenetic information of the strains is needed, and this is called polyphasic taxonomy. Phenotypic information is derived from main physical characteristics such as morphology, staining properties, ultrastructure, chemotaxonomic markers, physiological properties, biochemical features, protein composition, and pathogenesis. Meanwhile, genotypic data are obtained from the nucleic acids (DNA and RNA) in the cell such as analysis of DNA–DNA hybridization (DDH), DNA G+C content and 16S rRNA gene sequencing[58,59,94]. Some other methods recently used for genomic taxonomy study include ribotyping and whole-genome analysis[95,96]. Phylogeny depicts biological entities that are connected through common descent. It can be species, genus or higher-level taxonomic groups. A phylogenetic tree is used for understanding not only the relationships among taxa (or sequences) but also their hypothetical related predecessors. Currently, most phylogenetic trees are constructed from molecular data such as DNA or protein sequences.

As mentioned above, the class of Actinobacteria is an essential group in both health and economy that consists of 29 orders and 62 families. In the effort of discovering drugs from Actinobacteria sources, polyphasic taxonomy is needed either for establishing reliable identification of antibiotics producing strains or as a dereplication tool to avoid redundancy in the selection of Actinobacteria species[97]. Microscopic morphology and chemotaxonomy are the primary characteristics employed to delineate the taxonomy of Actinobacteria at the genus and species levels. However, with the recent development in molecular analysis, some species that were inadequately positioned in particular taxonomic groups have recently been reclassified using molecular analyses[22].

1.3.1 Phenotypic analysis

Various morphologies can be found in Actinobacteria, which include coccoid (*Micrococcus*), rod-coccoid (*Arthrobacter*), irregular rods (*Propionibacterium*), fragmenting hyphal forms (*Nocardia*), and permanent and highly differentiated branched mycelia (*Streptomyces, Kitasatopora* and *Frankia*). The members of genus *Rhodococcus* form elongated filaments without producing a real mycelium. The members of genus *Corynebacterium* produce no mycelia. There is also a group that can break up its hyphae into flagellated motile elements (*Oerskovia*). Some members of *Mycobacterium* and *Rhodococcus* mostly do not form aerial hyphae[22,98,99].

Spore is one of the important markers in the taxonomy of Actinobacteria, although some Actinobacteria produce no spores, e.g., the members of *Bifidobacterium, Micrococcus,* and *Propionibacterium*. Spores may be produced on the substrate only (*Micromonospora*) or both on the substrate and the aerial mycelium (*Streptomyces*) either as single cells or in chains with several of length. In some groups, spores may be sheltered in specialized vesicles called sporangia (*Actinoplanes, Planomonospora, Planobispora, Dactylosporangium*, and *Streptosporangium*) and equipped with flagella for the motility of spores (*Actinoplanes* and *Actinosynnema*)[22,100,101].

Some Actinobacteria can produce various pigments, including melanin based on the medium that is used and the age of the culture. These may be red, yellow, orange, pink, brown, greenish-brown, blue, or black. Melanins are polymers with various structures that usually appear black or brown and are derived from oxidative polymerization of phenolic and indolic molecules. They are not necessary for the growth and development, but they can help the host for survival and competition to

other organisms. They can be used in taxonomic studies and have a similar character to soil humic substances[22]. Investigation of pigments including melanin produced by Actinobacteria and their features of the morphology such as substrate mycelia, aerial mycelia, and spores can be conducted by utilizing International *Streptomyces* Project (ISP) medium[102]. The observation of spores formed by Actinobacteria is conducted employing a scanning electron microscope (SEM)[103].

As mentioned above, physiological and biochemical properties are parts of phenotypic criteria in the taxonomy study. Physiological data useful for Actinobacteria classification determinations include growth temperature, pH value, salt tolerance, growth on sole carbon sources, antibiotic resistance and oxygen requirement whereas biochemical features of interest are carried out by enzymatic activity test[100,102,104–107].

In the classification of Actinobacteria, the analysis of chemical components in the cell such as the analysis of cell-wall amino acids, lipids, proteins, menaquinones, muramic acid types, and sugars, is required[22]. Some genera contain 2, 6-diaminopimelic acid (DAP) in their cell-wall structure, which has isomers, i.e., LL-DAP and meso-DAP. *Streptomyces*, *Sporichthya*, and *Intrasporangium* are the example of genera that have only LL-DAP. For meso-DAP, it can be found in the members of *Mycobacterium*, *Nocardia*, and *Pseudonocardia*. Hydroxy-diaminopimelic acid (OH-DAP), which is the derivative of DAP, is detected in *Micromonospora* strains. For whole-cell sugar patterns, *Streptomyces* species have no characteristic sugar pattern. *Mycobacterium*, *Nocardia*, and *Pseudonocardia* species have galactose and arabinose as their whole sugar pattern. *Microbispora* and *Streptosorangium* strains contain madurose, whereas the members of *Actinoplanes*, *Dactylosporangium*, and *Micromonospora*, possess xylose and arabinose[108].

Lipids, such as fatty acids, phospholipids, and menaquinones, have been used for chemotaxonomy of Actinobacteria. Fatty acids iso or anteiso C15:0, C15:0, or C17:0 have been found as the predominant fatty acid in *Rothia*, *Actinoplanes*, *Nocardiopsis*, *Amycolatopsis*, and *Streptomyces* species[106,109–112]. Diphosphatidyl glycerol, phosphatidyl inositol mannosides and phosphatidyl inositol are mostly found in Actinobacteria species. Many Actinobacteria possess phosphatidyl ethanolamine except the members of *Actinomadura*, *Corynebacterium*, *Microtetraspora*, and *Nocardioides*. Phosphatidylcholine can be found in *Actinomadura*, *Nocardia* and *Pseudonocardia* strains[113]. As for menaquinones, some *Corynebacterium* species

contain menaquinone MK-8(H2) as the predominant menaquinone. MK-8(H4) is the primary menaquinone system in *Nocardia* members. Many *Mycobacterium* strains possess menaquinone MK-9(H2) as the primary menaquinone system. The menaquinone compounds in some Streptomyces are dominated with MK-9(H6), whereas in *Actinomyces* species, MK-10(H4) is the main menaquinone they have[114].

Some taxonomy studies in Actinobacteria were reported analyzing ribosomal protein using matrix-assisted laser-desorption/ionization time-of-flight spectrometry (MALDI-TOF MS)[109,115,116]. Ribosomal proteins have also been used for taxonomy study of bacteria since their peaks in MALDI-TOF MS are dominant, they are rather conservative molecules in the context of evolution, and their spectra are also specific of a particular strain and undergo only a little change with different culture conditions[117].

1.3.2 Genomic and phylogenetic analysis

The genomic DNA G+C content is the ratio of guanine and cytosine within the total number of nucleotides in the genome. It is one of the taxonomic markers and can be employed for discriminating phenotypically similar microorganisms. It is also the primary characteristic of cellular DNA and is related with the amino acid composition of proteins, codon usage in mRNA, auxotrophy for specific bases, and other features of overall biological importance[118,119]. Class of Actinobacteria is encompassed mostly of Gram-positive bacteria with a high G+C content (>55 mol% in genomic DNA). Some members of *Corynebacterium*, however, have G+C content lower than 55% and even *Gardnerella* strains have G+C content which is less than 45%[23,120].

Ribosomal RNA has been used in the taxonomic study due to its availability in all self-replicating systems, ease of isolation, and its sequence changes very slowly. 16S ribosomal RNA (16S rRNA) is one of the ribosomal RNAs in bacteria. 16S rRNA gene is used as the backbone for the classification of bacteria because it is ubiquitous, functionally stable, highly conserved and poorly subject to horizontal gene transfer (HGT). In the recent decade, comparative analysis of the 16S rRNA sequence, which has a size around 1,500 nucleotides, has been employed to study prokaryote phylogeny. In the 16S rRNA gene sequence analysis, the cut-off of identity values for determining novel genus is 95%, whereas it is 98.7% for novel species (previously it was 97%), when the 16S rRNA gene sequence of the tested bacterial strains are compared with their phylogenetically closest neighbours with validly published

names[59,121–123]. In the case of *Streptomyces* species, previous studies found that there are some novel *Streptomyces* species, which have the similarity value of 16S rRNA gene sequence comparison 99.1-99.9%[124–128].

Ribotyping is a genetic fingerprinting method, which uses digested genomic DNA, separating it by gel electrophoresis, and hybridizing it with ribosomal DNA (rDNA) probe by Southern blotting technique. The restriction enzymes that are used can be *Eco*RI, *Pst*I, *Pvu*II, *Bam*HI, *Cla*I and *Hind*III. The complete ribosomal operon from *Escherichia coli* is employed as the rDNA probe. The resulted pattern is a genetic fingerprint, which is useful to discriminate bacterial strains. Many bacterial species can be delineated with Ribotyping method, including Actinobacteria[95,129].

DNA–DNA hybridization is a commonly used technique to evaluate the genetic relationship between bacterial strains and is still known as the 'gold standard' principle for species delineation of prokaryotes. The tested bacterial strains are indicated as distinct species when their DDH values are ≤ 70%. DDH, however, has some shortcomings such as the cut-off values are not pertinent to all prokaryote genera, determining DDH values needs special facilities therefore the method is not available in many laboratories, and it is a laborious and expensive method that has shortage of reproducibility and is challenging to create a comparative reference database progressively from DDH data[59].

Multi-locus sequence analysis has been used for delineation some bacterial species in several genera, including members of Actinobacteria such as *Mycobacterium*, *Streptomyces* and *Kitasatospora*. By using the concatenation of partial gene sequences from five house-keeping genes, the closely related species of *Streptomyces* can be discriminated. These five house-keeping genes are *atpD* (ATP synthase F1, beta subunit), *gyrB* (DNA gyrase B subunit), *recA* (recombinase A), *rpoB* (RNA polymerase, beta subunit) and *trpB* (tryptophan synthase, beta subunit). This method is reproducible, can allow creating cumulative databases, and is comparable to DDH. The five-gene MLSA distance of 0.007 corresponds to a DDH value of 70%, which means that this value could be employed as the species cut-off for the whole *Streptomyces* genus. The sequences derived from the concatenated genes can also be used for generating phylogenetic tree [24,130,131].

Whole-genome data has also been used in bacterial taxonomy (including Actinobacteria), i.e., by aligning and comparing whole-genome sequences between two genome sequences from the closely related strains. The term overall genome relatedness index (OGRI) is coined to represent any measurements showing how identical two tested genome sequences are. The most commonly used algorithm for taxonomic investigations is average nucleotide identity (ANI). When comparing to DDH, 95-96% ANI value is equivalent to 70% DDH. It means ANI value can be employed to delineate novel species in bacteria[96,132,133].

1.4 Dereplication and isolation of natural products from Actinobacteria

Discovering of bioactive molecules from Actinobacteria sources nevertheless requires substantial investments in technical equipment, time and human resources. Rapid identification of known substances, which is called dereplication, is one of the important strategies to focus the efforts on the finding of novel bioactive compounds. The term of dereplication nowadays, however, is a not so univocal and has developed in the past few years in different ways. There are many dereplication methods developed to enhance the success of natural product discovering programs[134,135].

Dereplication approach includes some methods, e.g., biological screening processes, LC-MS (liquid chromatography-mass spectrometry) techniques with the combination of MS libraries and databases, NMR (nuclear magnetic resonance) spectroscopy, and HTS (high throughput screening) technology. Taxonomic identification using 16S rRNA gene sequence has been used since the early of the 2000s as a dereplication tool. Bioactivity-guided assays with combination LC-MS have also been employed for dereplication approach[97].

Hubert *et al.*[135] reviewed dereplication strategies in natural product research. They classified dereplication approaches into five categories, i.e., DEREP1, DEREP2, DEREP3, DEREP4, and DEREP5. Identification of the major compounds in a single extract is the activity in DEREP1. Systematically biological assay is included in DEREP2 (acceleration of activity-guided fractionation). DEREP3 is chemical profiling of crude extract collections. Chemical profiling of target compounds is conducted in DEREP4. The last, DEREP5 is the dereplication with taxonomic identification of microbial strains. Dereplication of natural products from Actinobacteria sources, therefore, may be conducted by using 16S rDNA analysis and with the combination of LC-MS-bioassay method.

Isolation of natural products from Actinobacteria may be carried out following the bioassay-guided isolation strategies that connect information on the chemical profiles of extracts and the active fractions. Extraction procedures employ organic solvents of different polarity, water and the mixtures of them or using solid-phase extraction (SPE) method[136]. Bioactivity assays can be done with antibacterial, antifungal, anticancer, or antiviral activity, e.g., antihepatitis virus, which is described briefly below. The antimicrobial test may be conducted either by using the zone inhibition test[137] or serial dilution test with 96-well plate[138]. The further isolation process needs preparative thin layer chromatography (TLC) or column chromatographic methods. Finally, for the structure elucidation of the isolated compound, high resolution mass spectrometry (HR-MS) and NMR spectroscopy is employed[136,139]. HR-MS can provide accurate mass measurements that is needed for determination of chemical formula resulting high confidence in the structural elucidation[139]. As for NMR, it is used for determining the molecular structure of the compound[140].

1.5 Hepatitis

Hepatitis is a disease identified by inflammation of the liver tissue and is caused mostly by viruses. There are five types of hepatitis viruses, i.e., type A, B, C, D, and E. Hepatitis A and E are usually caused by consuming contaminated food or water, while hepatitis B happens frequently through contact with infected bodily fluids like blood or semen. Hepatitis C virus is a blood-borne virus that is mostly transmitted by the usage of sharing needles from person to person. As for hepatitis D virus, it is spread with the infectious blood and the disease occurs only among the patients who have been infected by hepatitis B virus. Hepatitis B, C, and D plague more than half a billion people throughout the world and cause more than a million deaths annually[141].

Hepatitis A virus is a nonenveloped RNA with 7.5-kb genome size and belongs to the genus *Heparnavirus* of the *Picornaviridae* family[142]. Hepatitis B virus is a DNA virus and a member of the genus *Orthohepadnavirus* in the *Hepadnavidae* family[143]. Hepatitis C virus (HCV) is a member of the genus *Hepacivirus* in the family *Flaviviridae*. It is a positive single-stranded RNA virus with circa 9.6 kb size. It mainly propagated in the hepatocyte cytoplasm and mostly causes acute or chronic hepatitis C (CHC)[144]. Hepatitis D virus is an RNA virus and belongs to *Deltavirus* genus in the *Deltaviridae* family. It is a satellite virus of HBV because its incapability of infection in the absence of Hepatitis B virus[145]. Hepatitis E virus (HEV) is a single

stranded and a positive RNA virus. It belongs to the *Orthohepevirus* genus in the *Hepeviridae* family. Its genome size is 7.2 kb and it is the primary cause of global enterically transmitted hepatitis[146].

1.6 Previous work

In the course of Actinobacteria investigation from the neglected habitat sources, strain ASO4wet was isolated from the sponge in a deep sea. In December 2014, the Team of Prof. Dr. Peter Schupp (University of Oldenburg) took samples from marine sediment from 1092 m depth in the North Atlantic Ocean during an expedition with a new marine research vessel called Sonne. Study of 16S rRNA gene sequences revealed that strain ASO4wet is a member of the genus *Streptomyces* and exhibited the closest similarities to *S. karpasiensis* (98,94%), *S. glycovorans* (98%) and *S. abyssalis* (98%). DNA-DNA hybridization (DDH) analyses between strain ASO4wet and its closest related type strains indicated that strain ASO4wet is a distinct species from its compared closest species. Some studies have been conducted regarding to the novel description of the novel species for strain ASO4wet, including morphology and biochemical comparison, DDH, ribotyping, and MALDI-TOF analysis. From these studies, it is known that strain ASO4wet is different based on the morphologic, biochemical, and genomic characteristic from its close neighbours. However, chemotaxonomy study such as fatty acid, phospholipid, cell-wall amino acid, and whole-cell sugar analysis, of isolate ASO4wet has not yet been conducted and therefore, it needs to be carried out in order to fulfil the requirement of polyphasic taxonomy of strain ASO4wet[147].

1.7 Aim of study

The objectives of the research are:

1) Isolation of Actinobacteria from Indonesia
2) Cultivation and induction of secondary metabolite production (by using different media)
3) Extraction and screening of bioactivity, including antimicrobial and antiviral activity
4) Analysis, isolation and structure elucidation of metabolites produced by isolated Actinobacteria
5) Taxonomic study of selected Actinobacteria strains including strain ASO4wet

2 Materials and Methods

2.1 Materials

2.1.1 Media

All of the media were sterilized for 20 min at 121°C.

Table 4. List of media used in this study.

Medium	Composition or Manufacturer	Amount	pH
ISP medium (International *Streptomyces* Project)			
ISP2/ Yeast malt agar	Malt extract	10.0 g/l	7
Maintenance and taxonomy	Yeast extract	4.0 g/l	
	Glucose	4.0 g/l	
	Agar	15.0 g/l	
	Deionized Water	1000 ml	
ISP3/ Oat meal agar	Oatmeal (Quaker white oats)	20.0 g/l	7.2
Maintenance and taxonomy	Agar	18.0 g/l	
	Deionized Water	1000 ml	
	Trace salt solution	1 ml	
Trace salt solution ISP3	$FeSO_4$ x 7H2O	0.1 g	
	$MnCl_2$ x 4H2O	0.1 g	
	$ZnSO_4$ x 7H2O	0.1 g	
	Deionized water	100ml	
ISP 4	Soluble starch	10.0 g/l	7.3
Maintenance and taxonomy	$(NH_4)_2SO_4$	2.0 g/l	
	K_2HPO_4	1.0 g/l	
	$MgSO_4$ x 7H2O	1.0 g/l	
	NaCl	1.0 g/l	
	$CaCO_3$	2.0 g/l	
	Agar	20.0 g/l	
	Deionized Water	1000 ml	
ISP5	L-Asparagine	1.0 g/l	7.2
Maintenance and taxonomy	Glycerol	10.0 g/l	
	K_2HPO_4	1 g/l	
	Trace salt solution	1ml/l	
	Agar	20 g/l	
	Deionized Water	1000 ml	
Trace salt solution ISP5	1,0 g FeSO4 x 7 H2O	1.0 g	
	1,0 g MnCl2 x 4 H2O	1.0 g	
	1,0 g ZnSO4 x 7 H2O	1.0 g	

	Deionized water	100 ml	
ISP6 / Peptone Iron Agar Production of melanoid pigment	Peptone	15.0 g/l	7.2
	Proteose Peptone	5.0 g/l	
	Ferric ammonium citrate	0.5 g/l	
	Sodium glycerophosphate	1.0 g/l	
	Sodium thiosulfate-5-hydrate	0.126 g/l	
	Yeast extract	1.0 g/l	
	Agar	20 g/l	
	Deionized Water	1000 ml	
ISP7/ Oat meal agar Production of melanoid pigment	Glycerol	15 g/l	7.3
	L-Tyrosine	0.5 g/l	
	L-Asparagine	1.0 g/l	
	K_2HPO_4	0.5 g/l	
	NaCl	0.5 g/l	
	$FeSO_4$ x 7 H2O	0.01 g/l	
	Trace Salt solution (5343)	1.0 ml/l	
	Agar	20.0 g/l	
	Deionized water	1000 ml	
GYM/ *Streptomycetes* Medium Maintenance and revitalization	Glucose	4.0 g/l	7.2
	Yeast extract	4.0 g/l	
	Malt extract	10.0 g/l	
	$CaCO_3$	2.0 g/l	
	Agar	12 g/l	
	Deionized Water	1000 ml	
GYM + ASW (Artificial Sea Water)	GYM medium	1000 ml	7.2
	Coral reef salt “Coral Ocean”, ATI	39 g	
Synthetically Suter Medium Production of melanoid pigment	Glycerol	15.0 g/l	7.2
	Tyrosine	1.0 g/l	
	L-arginine	5.0 g/l	
	L-glutamic acid	5.0 g/l	
	L-methionine	0.3 g/l	
	L-isoleucine	0.3 g/l	
	K2HPO4	0.5 g/l	
	MgSO4 x 7 H2O	0.2 g/l	
	Trace element solution 2 (5341)	1.0 ml/l	
	Agar	20 g/l	
	-Control medium is prepared without tyrosine		
Basal medium for carbohydrate utilization	$(NH_4)_2SO_4$	2.64 g/l	7.3
	KH_2PO_4	2.38 g/l	
	K_2HPO_4	4.31 g/l	
	$MgSO_4$ x 7 H2O	1.0 g/l	
	Agar	15.0 g/l	

	Trace element solution 3 (5342)	1.0 ml/l	
	Deionized Water	1000 ml	
5341	$CuSO_4$ x 5 H2O	10.0 g/l	
Trace element solution 2	$CaCl_2$ x 2 H2O	10 g/l	
	$FeSO_4$ x 7 H2O	10 g/l	
	$ZnSO_4$ x 7 H2O	10 g/l	
	$MnSO_4$ x 7 H2O	40 g/l	
	Deionized water	1000 ml	
5342	$CuSO_4$ x 5 H2O	0.64 g/l	
Trace element solution 3	$FeSO_4$ x 7 H2O	0.11 g/l	
	$ZnSO_4$ x 7 H2O	0.15 g/l	
	$MnCl_2$ x 4 H2O	0.79 g/l	
	Deionized water	1000 ml	
5343	$FeSO_4$ x 7 H2O	1 g/l	
Trace element solution 4	$ZnSO_4$ x 7 H2O	1 g/l	
	$MnCl_2$ x 4 H2O	1 g/l	
	Deionized water	1000 ml	
Sodium chlorite tolerance	Casein peptone	10.0 g/l	7.0
	Yeast extract	5.0 g/l	
	Agar	20 g/l	
	Deionized Water	1000 ml	
5006	Sucrose	3.0 g/l	7.2
sterility control	Dextrin	15.0 g/l	
	Meat extract	1.0 g/l	
	Yeast extract	2.0 g/l	
	Tryptone soy broth	5.0 g/l	
	NaCl	0.5 g/l	
	K_2HPO_4	0.5 g/l	
	$MgSO_4$ x 7 H2O	0.5 g/l	
	$FeSO_4$ x 7 H2O	0.01 g/l	
	Agar	20 g/l	
	Deionized Water	1000 ml	
5336	Soluble starch	10.0 g/l	7.3
Actinobacteria isolation medium	Casein (Pepton Typ M)	1.0 g/l	
	K_2HPO_4	0.5 g/l	
	$MgSO_4$ x 7 H2O	5.0 g/l	
	Agar	20.0 g/l	
	Deionized water	1000	
5336 + Cyclo + NA	Sterilized 5336 medium	1000 ml	7.3
Actinobacteria isolation medium	Cyclohexamide solution (50 mg/ml in methanol)	2 ml	
	Nalidixic acid solution (25 mg/ml in 0.2 M NaOH)	1 ml	

Medium 5254	Glucose	15.0 g/l	7.0
Metabolite production	Soymeal	15.0 g/l	
	Corn steep liquor	5.0 g/l	
	$CaCO_3$	2.0 g/l	
	NaCl	5.0 g/l	
	Deionized water	1000 ml	
Medium 5294	Starch (soluble)	10.0 g/l	7.2
Metabolite production	Yeast extract	2 g/l	
	Glucose	10 g/l	
	Glycerol	10 g/l	
	Corn steep liquor	2.5 g/l	
	Peptone (Marcor S)	2.0 g/l	
	NaCl	1 g/l	
	CaCO3	3.0 g/l	
	Deionized water	1000 ml	
Medium 5294HG-S	Yeast extract	2 g/l	7.2
Metabolite production	Glucose	20 g/l	
	Glycerol	10 g/l	
	Corn steep liquor	2.5 g/l	
	Peptone (Marcor S)	2.0 g/l	
	NaCl	1.0 g/l	
	CaCO3	3.0 g/l	
	Deionized water	1000 ml	
5254 + ASW	5254 medium	1000 ml	7.0
(Artificial Sea Water)	Coral reef salt “Coral Ocean”, ATI	39 g	
5294 + ASW	5294 medium	1000 ml	7.2
(Artificial Sea Water)	Coral reef salt “Coral Ocean”, ATI	39 g	
SYP medium	Starch (soluble)	10 g	7.0
	Yeast extract	4 g	
	Peptone (Marcor S)	2 g	
	Deionized water	1000 ml	
Middelbrock Broth medium	Becton, Dickinson and Company, France		
Müller-Hinton Bouillon (MHB)	Carl Roth GmbH + Co.KG, Germany		
Myc medium	Phytone peptone	0.1 g	7.0
	Glucose	0.1 g	
	HEPES	11.9 g	
	Deionized water	1000	
Trypticase soy broth	Becton, Dickinson and Company, France		
Tryptone soy broth	Oxoid		

2.1.2 Chemicals

Table 5. List of chemicals used in this study.

Chemical	Manufacturer
1 kb DNA ladder	BioLabs
Acetic acid	Carl Roth
Acetone	J.T. Baker
Acetonitrile	J.T. Baker
Agarose	Gibco BRL
Ammonium acetate	Carl Roth
Ammonium iron(III)citrate ($(NH_4)_5Fe(C_6H_4O_7)_2$)	Carl Roth
Ammonium Sulfate $(NH_4)_2SO_4$	Merck
Aniline phthalate spray solution for TLC	Carl Roth
Anisaldehyde solution spray reagent	Sigma-Aldrich
Arabinose	Merck
Bacto Agar®	Becton Dickinson
Calcium carbonate ($CaCO_3$)	Panreac Appli Chem
Casein (Pepton Typ M)	Marcor
Cellulose	Serva, Heidelberg
Copper(II) sulfate ($CuSO_4$ x 5 H_2O)	Merck
Corn steep liquor	Schering
Dimethylsulfoxid (DMSO)	Carl Roth
Dulbecco´s modified Eagle´s medium (DMEM)	Bio Whittaker, Walkersville, MD
Cyclohexamide	Serva
Ethanol	J.T. Baker
Ethyl acetate	J.T. Baker
Ethylenediaminetetraacetic acid (EDTA)	Honeywell
Ferric ammonium citrate	Merck
Fetal bovine serum (FBS)	FBS, JRH Bioscience, Lenexa, KS
Formic acid	Sigma- Aldrich
Fructose	Carl Roth
Glucose	Carl Roth
Glycerol	Carl Roth
HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Carl Roth
Hydrochloric acid (HCL)	Carl Roth
Inositol (Ino)	Merck
Iron(II) sulfate ($FeSO_4$ x 7 H_2O)	Honeywell

Iron(III) chloride ($FeCl_3$ x $6H_2O$)	Merck
Iron(III) citrate ($FeC_6H_6O_7$ x H_2O)	Merck
Iron(III) ethylenediaminetetraacetic acid (Fe EDTA)	Fluka
Jump Start Taq Ready Mix	Sigma- Aldrich
K_2HPO_4	Merck
KH_2PO_4	Carl Roth
L-Arginine	Panreac Appli Chem
L-Asparagine	Panreac Appli Chem
L-Glutamic acid	Merck
L-Isoleucine	Fluka
L-Methionine	Panreac Appli Chem
L-Tyrosine	Merck
Malt Extract	Carl Roth
Meat Extract	Carl Roth
Molybdatophosphoric acid spray solution for TLC	Merck
Molybdenum blue spray reagent	Sigma
$MgSO_4$ x $7H_2O$	Carl Roth
$MnCl_2$ x $4H_2O$	Merck
NaCl	Carl Roth
Nalidixic acid	Carl Roth
α-Naphtol	Merck
α-Naphtol spray reagent composition: 15% α-naphtol in ethanol (10.5 ml) Concentrated H_2SO_4 (6.2 ml) Ethanol (40.5 ml) Deionized water (4 ml)	
Ninhydrin spray reagent	Sigma
Peptone	Becton Dickinson
Peptone S	Marcor
Phytone peptone	Carl Roth
Potassium chloride (KCl)	Sigma- Aldrich
Proteose Peptone	Becton Dickinson
Reef salt “Coral Ocean”	ATI, Hamm
Sodium glycerophosphate	Fluka
Sodium thiosulfate-5-hydrate	Merck
Soluble starch	Carl Roth
Sucrose	Merck

Sulfuric acid (H_2SO_4)	Merck
Water (nuclease free)	Carl Roth
Yeast extract	Carl Roth
$ZnSO_4$ x $7H_2O$	Merck

2.1.3 Source of samples

Table 6. List of samples collected from Indonesia for Actinobacteria isolation.

Sample code	Location of source	Type of sample
1808	Malang, East Java	Soil
1899 (FK1)	Forest low altitude, Kendari, Southeast Sulawesi	Soil
1900 (FK2)	Forest low altitude, Kendari, Southeast Sulawesi	Soil
1901 (FK3)	Forest low altitude, Kendari, Southeast Sulawesi	Soil
1902 (FK4)	Forest low altitude, Kendari, Southeast Sulawesi	Soil
1903 (MK5)	Mangrove, Kendari, Southeast Sulawesi	Soil
1904 (MK6)	Mangrove, Kendari, Southeast Sulawesi	Soil
1905 (MK7)	Mangrove, Kendari, Southeast Sulawesi	Soil
1906 (MK8)	Mangrove, Kendari, Southeast Sulawesi	Soil
1907 (GC9)	Cimahi, West Java	Soil
1929	Beach, Bali	Sand
1930	Beach, Bali	Dead barnacle
1931	Beach, Bali	Algae red
1932	Beach, Bali	Sand from reef
1933	Beach, Bali	Sand
1934	Beach, Bali	Sand
1935	Beach, Bali	Sand
1936	Beach, Bali	Green algae
1937	Beach, Bali	Brown algae
1938	Beach, Bali	Sand from reef
1939	Beach, Bali	Sand from reef
1940	Beach, Bali	Grass
1941	Beach, Bali	Sand from reef
1942	Beach, Bali	Sand

1943	Beach, Bali	Red stone from reef
1944	Beach, Bali	Algae
1945	Cultural Park, Bali	Sand
1946	Cultural Park, Bali	Sand
1947	Cultural Park, Bali	Sand
1948	Cultural Park, Bali	Sand
1949	Cultural Park, Bali	Soil
1950	Cultural Park, Bali	Soil
1951	Cultural Park, Bali	Soil
1952	Cultural Park, Bali	Soil
1953	Cultural Park, Bali	Soil
1955	Lava beach, Bali	Black sand
1956	Lava beach, Bali	Black sand
1957	Lava beach, Bali	Black sand
1958	Lava beach, Bali	Black sand
1959	Lava beach, Bali	Green algae
1960	Lava beach, Bali	Black sand
1961	Lava beach, Bali	Dead wood
1962	Lava beach, Bali	Algae
1963	Beach, Bali	Sand
1964	Botanical Garden, Bali	Soil with plant residue
1965	Botanical Garden, Bali	Soil with plant residue
1966	Botanical Garden, Bali	Soil with plant residue
1967	Botanical Garden, Bali	Soil with plant residue
1968	Botanical Garden, Bali	Soil with plant residue
1969	Botanical Garden, Bali	Soil with plant residue
1970	Botanical Garden, Bali	Bark
1971	Botanical Garden, Bali	Termite soil
1972	Botanical Garden, Bali	Lichen grey
1973	Botanical Garden, Bali	Fern residues
1974	Botanical Garden, Bali	Swpr
1975	Botanical Garden, Bali	Soil with moss
1976	Ecology Park, Bogor, West Java	Dry grass
1977	Ecology Park, Bogor, West Java	Bark
1978	Ecology Park, Bogor, West Java	Soil
1979	Ecology Park, Bogor, West Java	Dead wood
1980	Ecology Park, Bogor, West Java	Dry leaves

1981	Botanical Garden, Bogor, West Java	Exuvia Cicada?
1982	Botanical Garden, Bogor, West Java	Soil
1983	Botanical Garden, Bogor, West Java	Termite soil
1984	Botanical Garden, Bogor, West Java	Soil
1985	Botanical Garden, Bogor, West Java	Hypoxylon?
1986	Botanical Garden, Bogor, West Java	Bark
1987	Botanical Garden, Bogor, West Java,	Hypoxylon?
1988	Botanical Garden, Bogor, West Java	Bark dimocarpus
1989	Botanical Garden, Bogor, West Java	Lake soil
1990	Botanical Garden, Bogor, West Java	Lake soil
BL	Beach, Lampung	Sand
BLS	Beach, Lampung	Seaweed
BKB	Beach, West Kalimantan	Sand
BP	Beach, Papua	Sand
BB	Beach, Bali	Sand
MKB	Mangrove, West Kalimantan	Soil
2111	Mangrove, Jakarta	Soil
2112	Mangrove, Jakarta	Soil
2113	Mangrove, Jakarta	Dead wood
2114	Mangrove, Jakarta	Bark
2115	Mangrove, Jakarta	Soil
2116	Mangrove, Jakarta	Bark
2117	Mangrove, Jakarta	Dead wood
2118	Mangrove, Jakarta	Soil
2119	Mangrove, Jakarta	Soil
2120	Mangrove, Jakarta	Dead tree fungi
2121	Mangrove, Jakarta	Soil
2122	Mangrove, Jakarta	Dead wood
2123	Mangrove, Jakarta	Soil
2124	Mangrove, Jakarta	Soil
2125	Mangrove, Jakarta	Soil
2126	Mangrove, Jakarta	Dead leaves
2127	Mangrove, Jakarta	Soil
2128	Mangrove, Jakarta	Soil
2129	Mangrove, Jakarta	Soil
2130	Botanical Garden, Bogor	Wood flour
2131	Botanical Garden, Bogor	Dead roots

2132	Botanical Garden, Bogor	Moss
2133	Botanical Garden, Bogor	Soil
2134	Botanical Garden, Bogor	Dead wood
2135	Botanical Garden, Bogor	Wood chips
2136	Botanical Garden, Bogor	Dead wood
2137	Botanical Garden, Bogor	Soil
2138	Botanical Garden, Bogor	Bark
2139	Botanical Garden, Bogor	Soil
2140	Botanical Garden, Bogor	Moss
2141	Botanical Garden, Bogor	Soil
2142	Botanical Garden, Bogor	Soil
2143	Botanical Garden, Bogor	Soil
2144	Botanical Garden, Bogor	Soil
2145	Botanical Garden, Bogor	Soil
2146	Botanical Garden, Bogor	Soil
2147	Botanical Garden, Bogor	Soil
2148	Botanical Garden, Bogor	Dead roots

2.1.4 Organisms

Table 7. Actinobacteria strains from Research Center for Biotechnology LIPI-Indonesia.

Strain	Location of source
BLH 12-3	Bitung, North Sulawesi, Indonesia
DHE 2-1	Enggano Island, Bengkulu, Indonesia
DHE 9-4	Enggano Island, Bengkulu, Indonesia
MAE 1-3	Enggano Island, Bengkulu, Indonesia
MAE 1-11	Enggano Island, Bengkulu, Indonesia
SHP 1-2	Enggano Island, Bengkulu, Indonesia
SHP 1-4	Enggano Island, Bengkulu, Indonesia
SHP 1-5	Enggano Island, Bengkulu, Indonesia
SHP 1-6	Enggano Island, Bengkulu, Indonesia
SHP 2-2	Enggano Island, Bengkulu, Indonesia
SHP 2-4	Enggano Island, Bengkulu, Indonesia
SHP 2-5	Enggano Island, Bengkulu, Indonesia
SHP 6-2	Enggano Island, Bengkulu, Indonesia
SHP 6-3	Enggano Island, Bengkulu, Indonesia
SHP 6-4	Enggano Island, Bengkulu, Indonesia

SHP 6-5	Enggano Island, Bengkulu, Indonesia
SHP 6-6	Enggano Island, Bengkulu, Indonesia
SHP 7-1	Enggano Island, Bengkulu, Indonesia
SHP 7-3	Enggano Island, Bengkulu, Indonesia
SHP 7-5	Enggano Island, Bengkulu, Indonesia
GKRL-2	Lampung, Indonesia
GKRL-3	Lampung, Indonesia
GKRL-4	Lampung, Indonesia
GBSL-9	Lampung, Indonesia

Table 8. Tested organisms for antimicrobial, antinematode, and cytotoxic activity.

Organism	Code or comment
Microbes	
Escherichia coli	DSM 1116
Escherichia coli TolC	Deficient of TolC (the outer membrane channel for multidrug efflux)
Chromobacterium violaceum	DSM 30191
Pseudomonas aeruginosa	DSM 19882
Staphylococcus aureus Newman	Obtained from PD Dr. Markus Bischoff, Saarland University Hospital, Homburg
Bacillus subtilis	DSM 10
Micrococcus luteus	DSM 1790
Mycobacterium smegmatis	ATCC 700084
Mucor hiemalis	DSM 2656
Pichia anomala	DSM 6766
Candida albicans	DSM 1665
Mammalian cells	
A-431	Human epidermoid carcinoma
A-549	Human lung carcinoma
Huh-7.5	Human hepatocellular carcinoma cell
HUVEC	Human umbilical vein endothelial cell
KB-3-1	Human cervix carcinoma
L-929	Murine fibroblast
MCF-7	Human breast adenocarcinoma
PC-3	Human prostate carcinoma

SK-OV-3	Human Caucasian ovary adenocarcinoma
Nematode	
Caenorhabditis elegans	Non-parasitic roundworm

2.1.5 Kits and enzymes

Table 9. The list of kits and enzymes used in the experiment.

Kits or enzymes	Usage	Manufacturer
Invisorb® Spin Plant Mini Kit	DNA extraction	Stratec/Invitek
NucleoSpin® Microbial DNA	DNA extraction	Macherey-Nagel
NucleoSpin® Gel and PCR Clean-up	PCR cleaning	Macherey-Nagel
JumpStart™ Taq ReadyMix™	PCR	Sigma-Aldrich
Nuclease P1 from *Penicillium citrinum*	DNA digestion	Sigma-Aldrich
Lysozyme from chicken egg white	Peptidoglycan lysis	Sigma-Aldrich
Proteinase K	Protein digestion in DNA extraction	Carl Roth
Bacterial alkaline phosphatase	Removing 3 and 5 phosphates from DNA and RNA	Thermo Fisher Scientific
API® ZYM	Semiquantitation of enzymatic activities	BioMérieux
API® Coryne	24-hour identification of Corynebacteria and coryne-like organisms	BioMérieux

2.1.6 Primers

Some primers are used for 16S rRNA analysis and multi locus sequence analysis (MLSA) (Table 10 and Table 11).

Table 10. The list of primers used for 16S rRNA analysis.

Primer	Sequence (5' -> 3')	Length (nt)	Position[a]	Reference
F27	AGA GTT TGA TCM TGG CTC AG	20	8-27	[122,148]
R518	CGT ATT ACC GCG GCT GCT GG	20	518-537	[149]
F1100	YAA CGA GCG CAA CCC	15	1100-1114	[150]
R1100	GGG TTG CGC TCG TTG	15	1100-1114	[150]

R1492	TAC GGY TAC CTT GTT ACG ACT T	22	1492-1513	[122,148]
R1525	AAG GAG GTG ATC CAG CCG CA	20	1522-1541	[150]

[a] Position number refers to the 16S sequence of *E. coli rrnB* (GenBank J01695[128]).

Table 11. The list of primers used for multi locus sequence analysis or MLSA.

Gene	Primer	Sequence (5' -> 3')	Length (nt)	Reference
atpD	*atpD*PF (amplification)	GTC GGC GAC TTC ACC AAG GGC AAG GTG TTC AAC ACC	36	[151]
	*atpD*PR (amplification)	GTG AAC TGC TTG GCG ACG TGG GTG TTC TGG GAC AGG AA	38	[151]
	*atpD*F (sequencing)	ACC AAG GGC AAG GTG TTC AA	20	[151]
	*atpD*R (sequencing)	GCC GGG TAG ATG CCC TTC TC	20	[151]
gyrB	*gyrB*PFA (amplification)	TC GAG GGT CTG GAC GCG GTC CGC AAG CGA CCC GGT ATG TA	40	[131,152]
	*gyrB*PAR (amplification)	GAA GGT CTT CAC CTC GGT GTT GCC CAG CTT CGT CTT	36	[131,152]
	*gyrB*FA (sequencing)	GCA AGC GAC CCG GTA TGT AC	20	[131,152]
	*gyrB*RA (sequencing)	GAG GTT GTC GTC CTT CTC GC	20	[131,152]
recA	*recA*PF (amplification)	CCG CRC TCG CAC AGA TTG AAC GSC AAT TC	29	[151]
	*recA*PR (amplification)	GCS AGG TCG GGG TTG TCC TTS AGG AAG TTG CG	32	[151]
	*recA*F (sequencing)	ACA GAT TGA ACG GCA ATT CG	20	[151]
	*recA*PR2 (sequencing)	GCS AGR TCG GGG TTG TCC TTS AGG AAG TTS CG	32	[152]
rpoB	*rpoB*PF (amplification)	GAG CGC ATG ACC ACC CAG GAC GTC GAG GC	29	[151]
	*rpoB*PR (amplification)	CCT CGT AGT TGT GAC CCT CCC ACG GCA TGA	30	[151]
	*rpoB*F1 (sequencing)	TTC ATG GAC CAG AAC AAC C	19	[151]
	*rpoB*R1 (sequencing)	CGT AGT TGT GAC CCT CCC	18	[151]

trpB	*trpB*PF (amplification)	GCG CGA GGA CCT GAA CCA CAC CGG CTC ACA CAA GAT CAA CA	41	[151]
	*trpB*PR (amplification)	TCG ATG GCC GGG ATG ATG CCC TCG GTG CGC GAC AGC AGG C	40	[151]
	*trpB*F (sequencing)	GGC TCA CAC AAG ATC AAC AA	20	[151]
	*trpB*R (sequencing)	TCG ATG GCC GGG ATG ATG CC	20	[151]

2.1.7 Equipment

Table 12. Equipment used in this work.

Equipment	Manufacturer
Centrifuge	Eppendorf Centrifuge 5804 R
Centrifuge	Eppendorf Centrifuge 5427 R
Clean Bench	Thermo Scientific Type MS 2020 1.2
CO_2 incubator	Thermo Scientific Heracell 150i CO2 Incubator
HPLC	Agilent 1260 Series; Aligent technology, USA
HPLC	Aligent 1100 series; Aligent technology, USA
HPLC column	XBrigde® C-18 3.5 µm, 2.1 mm x 100 mm, Waters
Incubator	Hereus Instruments Function Line
Light microscope	Zeiss Axio Sc pie. A1 microscope
MS (HRESIMS)	MaXis ESI-TOF-MS spectrometer (Bruker) equipped with an Agilent 1260 series RP-HPLC system
Multichannel pipette	RAININ 8-Kanal-Pipette ED P3 Plus 100-1200 µL
N2 dryer (plates)	MiniVap (porvair science)
Photometer	IMPLEN Nano Photometer UV / VIS Spektralphotometer
Pipettes	Eppendorf Research plus
Rotary evaporator	Heidolph Laborata 4003
Shaker	Pilot-Shake System Kühner RC-6-U
Shaker (plates)	Heidolph Titramax 1000
Thermocycler	Eppendorf Thermocycler Mastercycler gradient

Thin layer chromatography (TLC) plates cellulose	Merck
Thin layer chromatography (TLC) plates silica gel 60	Merck
UV detector	Herolab RH-5.1 darkroom hood + B-1393-3K7N camera
DNA sequencer	96-capillary-system from Applied Biosystems (ABI), 3730xl DNA Analyzer.

2.2 Methods

2.2.1 Sampling

The samples were taken by the members of the GINAICO (German-Indonesian Anti-Infective Cooperation) team: Dr. Tjandrawati Mozef, Dr. Kathrin I. Mohr, Dr. Enge Sudarman, and Senlie Octaviana from 2015 until 2017. The locations range from the western part of Indonesia until the eastern part of Indonesia such as Lampung in Sumatra Island; West Kalimantan in Kalimantan Island; Jakarta, Bogor, Cimahi, and Malang in Java Island; Bali Island; Kendari in Sulawesi Island; Papua Island (Figure 5).

2.2.2 Isolation of Actinobacteria

One gram of samples was heated at 60°C for 30 minutes to eliminate all the vegetative cells. After the temperature decreased, ten milliliters of sterile water were added to the samples. The mixtures then were serially diluted until a dilution of 1:1000. The samples were plated on agar medium 5336 supplemented with cycloheximide 100 (µg/ml) as antifungal agent[153] and incubated for 7-21 days at 30°C.

2.2.3 Extract Production

The precultures were cultivated in 100 ml of GYM or GYM+ASW medium in a 250 mL flask for five days at 30°C and 160 rpm on a rotary shaker then were transferred 1:10 to 100 mL of SYP, 5254, 5295, 5254+ASW, and 5294+ASW for metabolite production. The cultures were incubated further for 5-7 days at 30°C and 160 rpm. Afterward, 20 ml of the cultures were extracted with 20 ml of ethyl acetate and were centrifuged at 9000 rpm for 10 min to separate the phases between the two immiscible solvents. The upper phase was then transferred and evaporated with reduced pressure at 40°C. The extract was re-dissolved in 1 ml of ethyl acetate: acetone: methanol (1:1:1) [138,154].

Figure 5. Map of Indonesia with the sampling sites.The sampling locations are marked with a red circle. Credit picture from https://pasarelapr.com/images/map-of-indonesian-island/map-of-indonesian-island-6.png

2.2.4 Analysis of 16S rRNA sequences

Genomic DNA extraction was performed by using Invisorb Spin Plant Mini Kit (250) (Stratec Molecular, Germany). From the well-grown cell suspension, 500 µl were taken and centrifuged for 2 minutes at 11,000 rpm. The supernatant was discarded and the cell pellet was mixed with 100 µL of lysis buffer. The mixture was then incubated at 95 °C for 5 min and added again with 300 µL lysis buffer. Afterward, 20 µL protein kinase K were added and incubated for 30 min at 65°C. The remaining steps were conducted following the manufacturer's instruction.

Amplification of 16S rRNA genes and the purification of the PCR product were carried out using the methods described by Mohr *et al.*[155]. Two primers were used matching most of the known eubacterial orders on the positions 27 (forward) and 1492 (reverse) or 1525 (reverse). The reaction volume (50 µl) was created containing water (22 µl), primers (1 µl; 10 µM each), "Jump Start Ready Mix" or JSRM (25 µl) and template DNA (1 µl). The JSRM is a mixture of JumpStart Taq DNA polymerase, 99% pure deoxynucleotides and buffer in an optimized reaction concentrate. The PCR reaction was conducted in a Mastercycler Gradient (Eppendorf, Hamburg, Germany). The condition of reaction included: initial denaturation at 95°C (5 min); 34 cycles of denaturing at 94°C (30 s); annealing at 52°C (30 s); elongation at 72°C (120 s); final elongation at 72°C (10 min).

The PCR product was checked on the agarose gel (0.8%) and purified using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) following the manufacturer's protocol. The DNA sequencing was performed by using 96-capillary-system from Applied Biosystems (ABI), 3730xl DNA Analyzer. The primers for sequencing were F27, R518, F1100, R1100, and R1492 or R1525.

Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were performed using EzTaxon-e server (http://www.ezbiocloud.net/taxonomy)[156] and the sequences of the strains were aligned using the CLUSTAL W algorithm[157] from the MEGA X software package[158]. Phylogenetic analysis was achieved using the maximum-likelihood[159], maximum-parsimony[160] and neighbour-joining[161] algorithms from MEGA X[158]. The topologies of the inferred trees were evaluated by bootstrap analyses[162] based on 1000 replicates.

2.2.5 Morphology and melanin production

The morphological characteristics of the strains and the ability to produce melanin were examined by incubating them on ISP2 (International Streptomyces Project 2), ISP3, ISP4, ISP5, ISP6, ISP7 medium, SSM+T (synthetically Suter medium with tyrosine), and SSM-T (synthetically Suter medium without tyrosine)[102,163] after 14 days at 30°C. The colors of aerial and substrate mycelium, as well as the diffusible pigments, were determined by comparison with the RAL-code (https://www.ral-farben.de)[138]. Spore chain morphology and spore-surface ornamentation were observed after 14-30 days at 30°C on ISP 3 medium[102]. A block of agar (1 cm x 1 cm) containing bacteria with the spores was fixed in glutaraldehyde solution (5%)[164]. The sample was then critical-point-dried and gold–palladium-sputtered. Afterward, the morphology of the spores was observed by a Zeiss Merlin field emission scanning electron microscope (FESEM) with an Everhart-Thornley SE-detector and an Inlens-SEM detector in a 25:75% ratio applying the SEMSmart software version 5.05[109]. SEM analysis was done by Prof. Dr. Manfred Rhode, HZI Braunschweig.

2.2.6 Physiological and biochemical characteristic

2.2.6.1 Growth at different temperature and pH

Growth at different temperatures (15, 20, 25, 30, 37 and 44°C) on GYM medium agar and pH range (pH 2, 3, 4, 5, 6, 7, 8, 9 and 10) on ISP 2 medium liquid were observed after incubation for 14 days.

2.2.6.2 Carbon utilization

Carbon utilization was studied on basal medium for carbohydrate utilization or ISP 9 medium[102] containing 10 different carbon sources (1 %, w/v) such as glucose, arabinose, sucrose, xylose, inositol, mannitol, fructose, rhamnose, raffinose, and cellulose. For this study, 12-well flat bottom plate was used.

2.2.6.3 Sodium chloride tolerance

The sodium chloride tolerance study was based on the method of Kutzner *et al*.[165] with different concentration of NaCl: 0%, 2.5%, 5.0%, 7.5%, and 10%. In this examination, the 6-well flat bottom plate was employed.

2.2.6.4 Enzymatic assay

The enzyme profiles were studied by using API ZYM[166] and API Coryne strips[167]. At least five days old liquid culture of the bacteria were used for this study. The enzymes that can be detected with API ZYM and API Coryne system are listed in Table 13.

Table 13. The activities that can be analyzed by API ZYM and API Coryne.

API ZYM	API Coryne
Alkaline phosphatase	Nitrate reduction
Butyrate esterase (C4)	Pyrazinamidase
Caprylate esterase lipase (C8)	Pyrrolidony arylamidase
Myristate lipase (C14)	Alkaline phosphatase
Leucine arylamidase	β-glucuronidase
Valine arylamidase	β-galactosidase
Cystine arylamidase	α-Glucosidase
Trypsin	N-acetyl-β-glucosamidase
α-Chymotrypsin	Esculin (β-glucosidase)
Acid phosphatase	Urease
Naphtol-AS-BI-phosphohydrolase	Gelatine (hydrolysis)
α-Galactosidase	Glucose fermantation
β-Galactosidase	Ribose fermentation
β-Glucoronidase	Xylose fermentation
α-Glucosidase	Mannitol fermentation
β-Glucosidase	Maltose fermentation
N-acetyl-β-glucosaminidase	Lactose fermentation

α-Mannosidase	Sucrose fermentation
α-Fucosidase	Glycogen fermentation

2.2.6.5 Antibiotic susceptibility

Antibiotic susceptibility was evaluated by the disc-diffusion plate method[137] using antibiotic discs on ISP 2 agar medium incubated at 30°C for 7 days. One loop of bacteria from the agar plate or 100 µl of 5-7 days old liquid culture were diluted with 1 ml sterile water and 100 µl of the suspension was plated on the ISP 2 agar medium. Eight antibiotic discs were used: ampicillin (10 µg/disc), erythromycin (15 µg/disc), gentamycin 30 (µg/disc), tetracycline (30 µg/disc), vancomycin (30 µg/disc), cefotaxime (30 µg/disc), rifampicin (5 µg/disc), and penicillin G (6 µg/disc).

2.2.7 Chemotaxonomy

2.2.7.1 Freeze-dried cells preparation

For chemotaxonomy study, freeze-dried cells were used instead of the wet biomass to ease the extraction of chemical substances from the cells. The biomass of a 5-7 days old grown culture in the medium GYM, ISP2 or TSB was collected by centrifuge at 9000 rpm for 10 minutes. The pellet was washed three times with demineralized water and centrifuged three times at 9000 rpm for 10 minutes. The wet biomass was freeze-dried for two days.

2.2.7.2 Cell wall amino acid analysis

The cell wall amino acid analysis was carried out as described by Staneck and Roberts[168]. Freeze-dried cells (3 mg) and 200 µl of 20% HCl or 6N HCl were added to a 4 ml brown glass vial. The vial was tightly closed with the lid and heated at 100°C for 18h. The mixture was cooled down and filtered by Whatman filter paper. The filtrate was evaporated at 100°C. Demineralized water (500 µl) was added and the liquid was dried again at 100°C to remove the remains of HCl. The dried extract was dissolved with 100 µl demineralized water. The amount of 2 µl was applied for TLC (Thin Layer Chromatography) analysis using an aluminium-backed cellulose plate. The mobile phase was methanol- demineralized water -6 N HCl-pyridine (80:26:4: 10, vol/vol). Standard amino acid (1 µl, 5 mg/ml) was diaminopimelic acid. Spots of amino acids were visualized by spraying with ninhydrin and heating at 100°C for 3 min.

2.2.7.3 Whole-cell sugar analysis

The whole-cell sugars analysis were prepared following the method of Staneck and Roberts[168]. Freeze-dried cells (25 mg) and 1.5 ml of 2.5% H_2SO_4 or 1N H_2SO_4 were added to a 4 ml brown glass vial. The vial was tightly closed with the lid and heated at 100°C for 2 h. After cooling down, the hydrolysate was transferred to a 15-ml conical centrifuge tube, and saturated barium hydroxide ($Ba(OH)_2$. H_2O = 3 gr/10 ml) was added carefully until the pH reached 5.2 and 5.5 (determined with pH paper). The precipitate was removed by centrifugation (9000 rpm, 5 min) and discarded. The supernatant fluid was evaporated at 35°C with nitrogen flow, and the residue was redissolved with 100 µl demineralized water. The amount of 2 µl was applied for TLC analysis using aluminium-backed cellulose plate. The mobile phase was n-butanol-demineralized water-pyridine-toluene (10:6:6:1, vol/vol). Two standards containing mixtures of sugar were employed because of the closely Rf value (retention factor) between the sugar. The first mixed sugar standard (0.5 µl, 10 mg/ml each) contained galactose, arabinose, and xylose. The second mixed sugar standard (0.5 µl, 10 mg/ml each) comprised rhamnose, mannose, glucose, and ribose. Spots of sugars were visualized by spraying with acid aniline phthalate and heating at 100°C for 5 min.

2.2.7.4 Menaquinone analysis

Menaquinones were extracted following the procedure performed by Minnikin *et al*.[169]. Freeze-dried cells (100 mg) were transferred into polytetrafluoroethene capped tube. The amount of 4 ml methanol-0.3% aqueous NaCl (90:10) and 4 ml petroleum ether were added to the tube. The suspension was mixed on tube rotator for 30 min. The upper phase was transferred to evaporator tube. The same amount of new petroleum ether was added again to the lower phase and mixed on tube rotator for 30 min. The upper layers were combined and evaporated at 30°C with reduced pressure until the volume remains about 1 ml. The extract was transferred to the 4 ml brown glass vial and dried at 30°C under N_2 flow. The dry extract was re-dissolved with 100 µl acetonitrile-isopropanol (65:35). The menaquinones were analyzed by high-performance liquid chromatography[103] equipped with diode-array detection and mass spectrometry (HPLC-DAD-MS). Here, high-resolution electron spray ionization mass spectrometry (HR-ESI-MS) data were recorded on a MaXis ESI-TOF-MS spectrometer (Bruker) equipped with an Agilent 1260 series RP-HPLC system. The HPLC system consisted of XBridge C18 column 2.1 x 100 mm, 1.7µm; solvent A was

isopropanol and solvent B was acetonitrile. The gradient system was 100% B for 3 min, 35% B in 5 to 15 min, and 50% B in 16-20 min. The flow rate was 0.6 mL/min, temperature of column was 40°C, and the UV-detection was at 270 nm. Molecular formula was calculated using the Smart Formula algorithm including the isotopic pattern (Bruker).

2.2.7.5 Polar lipid analysis

The polar lipids were extracted by the method of Minnikin *et al.*[170] and identified by two-dimensional thin-layer chromatography as described by Collins and Shah[111]. Freeze-dried cells (200 mg) were transferred into a polytetrafluoroethene capped tube. The amount of 8 ml Chloroform-MeOH (2:1) was added to the tube and mixed on tube rotator for 16 h. The suspension was filtered with filter paper and washed 2 times with 2 ml Chloroform-MeOH (2:1). The filtrate was combined to evaporation flask (25 ml volume size) and was evaporated with reduced pressure at 30°C. The extract was redissolved with 200 µl Chloroform-MeOH (2:1) and transferred to 0.3 ml brown glass vial.

Examination of polar lipids was conducted by two-dimensional thin-layer chromatography (TLC) using HPTLC Kieselgel $60F_{254}$ (Merck) plate (10 x 10 cm). The two-dimensional thin-layer chromatography was applied in this analysis. The first dimension was with chloroform/methanol/water (65:25:4 by volume) and in the second dimension was with chloroform/methanol/ acetic acid/water (80:12:15:4 by volume). For detecting all lipids, 10% molybdophosphoric acid in ethanol was sprayed to the plate and heated at 140°C for 15 min. Observation of specific lipid was carried out by spraying different reagents. Ninhydrin solution was used for detecting free amino groups (100°C, 4 min), molybdenum blue for phosphate-containing lipids, α-naphtol-H_2SO_4, anisaldehyde spray solution reagent (100°C, 10 min) for sugar-containing lipids, and Dragendorffs reagent for quaternary ammonium groups (Table 5).

2.2.7.6 Fatty acid analysis

Fatty acids were extracted, methylated and analyzed using Sherlock Microbial Identification (MIDI) system and the ACTIN version 6 database[171]. Freeze-dried cells (10 mg) were transferred into 13 x 100 culture tube. The amount of 1 ml saponification reagent (45 g NaOH, 150 ml methanol, 150 ml distilled water) was added to the tube.

The tube was sealed with teflon lined cap, vortexed and heated in a boiling water bath for 5 minutes. It was vortexed again for 5-10 minutes and returned to the boiling water bath to accomplish the 30-minute heating. The mixture was cooled into room temperature and added by 2 ml methylation reagent (325 ml HCl 6N, 275 ml methanol). The tube was capped, briefly vortexed, and heated for 10 minutes at 80°C. The mixture was cooled down into room temperature and added by 1.25 ml extraction reagent (200 ml hexane and 200 ml methyl tert.-butyl ether). The tube was recapped and tumbled on a rotator for 10 minutes. The tube then was uncapped and the aqueous phase (lower part) was pipetted out and discarded. About 3 ml of washing reagent (10.8 g NaOH dissolved in 900 ml distilled water) was added to the organic phase. The tube was recapped and tumbled for 5 minutes. About 2/3 of the organic phase (upper part) was pipetted into a GC vial for analysis. GC analysis was carried out with the conditions: using 25 m x 0.2 mm phenyl methyl silicone fused silica capillary column, the temperature program increased from 170°C to 270°C at 5°C per minute, employing flame ionization detector (FID), the carrier gas was hydrogen and the "make up" gas was nitrogen. Fatty acid analysis was conducted by Gabriele Pötter and Dr. Richard Hahnke, Leibniz Institut DSMZ-Braunschweig.

2.2.8 G+C (guanine + cytosine) contents determination

Genomic DNA for G+C contents analysis was prepared based on procedure conducted by Rong and Huang[172] with some modification. Twenty milliliters of well-grown culture were taken, centrifuged, washed three times with demineralized water and suspended in 10 ml STE buffer (75 mM NaCl, 25 mM Tris–HCl, 25 mM EDTA, pH 8.0) containing 5 mg lysozyme. Glass beads were added to the suspension and the mixture was vortexed to homogenize the cells. Afterwards, the suspension was incubated for 1 h at 37ºC in the tube rotator. The cells were completely lysed by adding 0.2 ml 20% (w/v) SDS and the lysate was further incubated at 55ºC for 2 h in the tube rotator. The DNA was extracted with the addition of chloroform (1:1, by volume) and the tube was centrifuged 9000 rpm for 10 minutes. The polar part (upper layer) was transferred carefully to the new tube and was added by 0.1 volume of 3 M sodium acetate (pH 5.5). The genomic DNA was precipitated with the addition 1 volume of isopropanol, rinsed two times with 70% ethanol, dried and dissolved in 1 ml of nuclease-free water.

The G+C content analysis was carried out adapting the method of Tamaoka and Komagata[173] and Mesbah *et al.*[118]. DNA solution containing 0.4-1.0 mg/ml in nuclease-free water was heated at 100°C for 10 minutes and cooled rapidly in an ice bath for 5 minutes. The denatured DNA solution was mixed with 16 µl of 30 mM sodium acetate buffer (pH 5.3), 2 µl of 20 mM $ZnSO_4$, and 2 µl of P1 nuclease (1 mg/ml in sodium acetate buffer; 340 U/ml). The mixture was incubated at 50°C for 1 h. Afterwards, 18 µl Tris Buffer 0.1 M pH 8.1 and 2 µl of Bacterial Alkaline Phosphatase (2.5 U/µl) was added to the mixture and incubated further at 65°C for 1 h. The hydrolysate was centrifuged at 11,000 rpm for 5 min and was transferred to 0.3 ml brown glass HPLC vial.

The HPLC system consisted of XBridge C18 column 2.1 x 100 mm, 1.7µm; solvent A was ammonium acetate buffer 5 mM and solvent B was acetonitrile/ ammonium acetate buffer 5 mM (95:5). The gradient system was 100% A to 95% A for 2 min, 95% A to 80% A for 4 min, 80% A to 60% A for 2 min and isocratic condition of 60% A for 2 min. The flow rate was 0.3 mL/min, the temperature of the column was 40°C, and the UV-detection was at 270 nm.

2.2.9 Multilocus sequence analysis (MLSA) for *Streptomyces* sp. SHP 1-2.

Genomic DNA extraction was performed by using Invisorb Spin Plant Mini Kit (250) (Stratec Molecular, Germany). From the well-grown cell suspension, 500 µl were taken and centrifuged for 2 minutes at 11,000 rpm. The supernatant was discarded and the cell pellet was mixed with 100 µL of lysis buffer. The mixture was then incubated at 95°C for 5 min and 300 µL lysis buffer were added. Afterwards, 20 µL protein kinase K was added and incubated for 30 min at 65°C. The remaining steps were conducted following the manufacturer's instruction.

Five housekeeping genes for multilocus sequence analysis (MLSA), *atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*, were used as reported previously by Guo *et al.*[151]. The gene *atpD* is responsible for expressing ATP synthase F1 beta subunit. The gene *gyrB* is for producing DNA gyrase B subunit. The gene *recA* is responsible for recombinase A expression. The genes *rpoB* and *trpB* are for preparing RNA polymerase beta subunit, and tryptophan synthase beta subunit, respectively[130]. The partial sequences of the five housekeeping genes were amplified and sequenced by using the primers based on the method of Guo *et al.*[151], Rong *et al.*[130], and Labeda *et al.*[24] (Table 11). The sequences of the five housekeeping genes were concatenated from head to tail and then aligned

using MUSCLE[174] in MEGA X[158]. The phylogenetic relationships were calculated using the maximum-likelihood[159], maximum-parsimony[160] and neighbour-joining[161] algorithms in MEGA X[158]. The MLSA distance was determined using Kimura 2-parameter distance[175]. The topologies of the inferred trees were evaluated by bootstrap analyses[162] based on 1000 replicates. The MLSA distance 0.007 is suggested previously equivalent to 70% DNA-DNA relatedness value[130]. That means the MLSA distance greater than 0.007 is considered as different species.

2.2.10 Bioactivity assay

2.2.10.1 Antimicrobial assay

Antimicrobial activity test was conducted by serial dilution in 96-well plates[16]. All the wells were filled with 150 µL of the microbial suspension (OD600 0.01 for bacteria and 0.05 for fungi). The first row was filled with another 130 µL of the suspension of the test trains. Then 20 µL of the compound were added to the first row and the sample was serially diluted with two-fold dilution to the next row until the last row. In the last row, 150 µL extra were discarded so that the volume of the suspension in all wells are the same (150 µL). The bacterial test strains were *Escherichia coli* DSM 1116, *Escherichia coli* TolC, *Chromobacterium violaceum* DSM 30191, *Pseudomonas aeruginosa* DSM 19882, *Staphylococcus aureus* Newman, *Bacillus subtilis* DSM 10, *Micrococcus luteus* DSM 1790, and *Mycobacterium smegmatis* ATCC 700084. The fungal test strains were *Mucor hiemalis* DSM 2656, *Pichia anomala* DSM 6766, and *Candida albicans* DSM 1665. The plates were incubated for 24-48 hours at 30°C or 37°C. A clear well was considered to show inhibition of microbial growth. Inhibition in A-B rows was considered as low activity, C-E rows were medium activity, and F-H rows were strong activity.

2.2.10.2 Nematicidal actitity

The nematicidal activity against *Caenorhabditis elegans* was determined by as described by Rupcic *et al.*[176]. The amount of 500 nematodes/ml in M9 buffer (NaCl 5 g/l, $Na_2HPO_4.2H_2O$ 7.5 g/l, KH_2PO_4 3 g/l, $MgSO_4.7H_2O$ 0.25 g/l, pH 7.2) and 66 µl of the tested compound were added in 24-well plate. The plates were then incubated at 20°C for 18 h. The dead and alive worms were counted using a microscope and a mechanic counter.

2.2.10.3 Cytotoxic assay

In vitro cytotoxicity assay was carried out based on the method of Landwehr *et al*.[154]. The cell lines for the assay were L-929 (murine fibroblast), KB-3-1 (human cervix carcinoma), A-549 **(**human lung carcinoma), PC-3 **(**human prostate carcinoma), MCF-7 **(**human breast adenocarcinoma), A-431 (human epidermoid carcinoma), SK-OV-3 (human Caucasian ovary adenocarcinoma), and HUVEC (human umbilical vein endothelial cell). The cells were grown at 37°C and 5% CO2 in Dulbecco's modified Eagle's medium (DMEM; high glucose) supplemented with 10% FCS (fetal calf serum). Then, 60 µl of serial dilutions of the test compounds were added to 120 µL of suspended cells (50,000 cells/mL) in the wells of 96-well plates. After five days of incubation, the inhibition of propagation (IC_{50}) was determined using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The cytotoxic assay was conducted by Wera Collisi, HZI-Braunschweig.

2.2.10.4 Antiviral assay

The antiviral assay was conducted according to Mulwa *et al*.[177]. Huh-7.5 cells (human hepatocellular carcinoma cells) stably expressing Firefly luciferase (Huh-7.5 Fluc) were inoculated with RLuc Jc1 reporter viruses and the extracts were given also to the cells. The cells were incubated at 37°C with 5% CO_2 supply. After 4 hours of incubation, the inoculum was discarded and the cells were washed with PBS (phosphate buffer saline) and were added with new medium without extracts. The medium for the cells was Dulbecco's modified minimum essential medium (DMEM, Life Technologies Manchester UK) containing 2 mM glutamine, 1 × minimum essential medium nonessential amino acids (MEM NEAA, Life Technologies), 100 µg/mL streptomycin, 100 IU/mL penicillin (Life Technologies), 5 µg/mL blasticidin and 10% FBS (fetal bovine serum). After 3 days of incubation, the infected cells were lysed. The reporter virus infection and the cell viability was determined by renilla luciferase and firefly luciferase activity, respectively. The measurement of luciferase activity was performed by using Berthold Technologies Centro XS3 Microplate Luminometer. The antiviral assay was conducted by Dimas F. Praditya and Prof. Dr. Eike Steinmann, TWINCORE-Hannover.

2.2.11 Fermentation, extraction and isolation of secondary metabolites from *Streptomyces* sp. SHP 1-2

The pre-culture of strain SHP 1-2 was grown in 250 mL Erlenmeyer flasks containing 100 mL GYM medium[154] for 5 days at 30ºC on a rotary shaker (160 rpm). The inoculum was transferred (1:10, by volume) into 500 mL Erlenmeyer flasks containing 200 mL 5294 medium[154]. A total of 10 L of liquid medium was cultivated at 30ºC for 5 days on the shaker with 160 rpm.

The fermentation broth (10 L) was harvested after 5 days of incubation and was centrifuged at 8500 rpm for 30 minutes. Afterwards, 5% (v/v) XAD-2 polymeric resin suspension was added to the filtrate and was stirred slowly for 16 hours in dark condition. The mycelium was extracted with 500 ml of acetone for three times. The extracts were combined and concentrated at 40°C with reduced pressure until the aqueous residue. This residue then partitioned with n-heptane and ethyl acetate respectively to yield n-heptane extract (63 mg) and ethyl acetate extract (781 mg). The solid residue from the mycelium was extracted again with ethyl acetate, evaporated at 40°C with reduced pressure, re-dissolved with methanol, and partitioned with n-heptane. The methanol extract then evaporated at 40°C under vacuum to give methanol extract (63 mg). The n-heptane extract (63 mg), ethyl acetate extract (781 mg), and methanol extract (63 mg) were combined and fractionated by a silica gel chromatography column eluting with gradient of CH_2Cl_2-ethyl acetate (from 100:0 to 0:100, by volume) to give eight fractions. Fraction 4 and fraction 7 were introduced separately to preparative reversed-phase HPLC using a C18 column with the gradient system (from 10% Acetonitrile: 90% Water to 100% Acetonitrile, by volume) to yield fraction 4.1 (35 mg) and fraction 7.1 (70 mg). The fermentation, extraction, and isolation of compound were carried out together by Dr. Rizna T. Dewi (RChem LIPI, Serpong), Gian Primahana, and Dr. Enge Sudarman (HZI-Braunschweig).

2.2.12 Fermentation, extraction and isolation of compound from *Amycolatopsis* sp. 196526CR

The pre-culture of strain 196526CR was grown in 250 mL Erlenmeyer flasks containing 100 mL GYM medium[154] for 5 days at 30ºC on a rotary shaker (160 rpm) and the inoculum was transferred (1:10, by volume) into 250 ml Erlenmeyer flasks containing 100 ml, 500 ml Erlenmeyer flasks containing 200 ml, or 1000 ml Erlenmeyer flasks containing 400 mL 5294HG-S medium (Table 4). A total of 15 L of liquid medium was cultivated at 30ºC for 7 days in the shaker 160 rpm.

The fermentation broth (15 L) was harvested after 7 days of incubation and centrifuged at 8500 rpm for 30 minutes. Afterwards, 5% (v/v) XAD-2 polymeric resin suspension were added to the filtrate and was stirred slowly for 16 hours in dark condition. The mycelium was extracted with acetone: methanol (1:1) for five times. The extracts were combined and concentrated at 40°C with reduced pressure until the aqueous residue. The aqueous residue was partitioned with ethyl acetate to yield ethyl acetate extract (6.1 g). The ethyl acetate extract was re-dissolved with 95% methanol and partitioned with n-heptane and dichloromethane to give n-heptane extract (3.3 g) and dichloromethane extract (551.5 mg). The dichloromethane extract was fractionated by an RP-18 chromatography column eluting with gradient of water (with formic acid 0.1%) and acetonitrile (with formic acid 0.1%) (from 40:60 to 0:100, by volume) to give fraction GRP17-19 (8.7 mg). Fraction GRP17-19 was further purified with RP-18 chromatography column eluting with gradient of water (with formic acid 0.1%) and acetonitrile (with formic acid 0.1%) (from 50:50 to 0:100, by volume) to yield fraction BDP61-63 (1.5 mg).

3 Results

3.1 Isolated Actinobacteria, 16S rRNA gene characterization, and bioactivity of the extracts

There were 196 Actinobacteria strains isolated from Indonesian samples. Twenty four of them were already isolated in Indonesia by Dr. Shanti Ratnakomala (Research Center of Biotechnology-LIPI). From 196 isolates, 84 strain were analyzed for their 16S rRNA gene during this work. There were 59 isolates characterized as *Streptomyces* and 25 isolates were non-*Streptomyces*. Some selected strains with with low percentage similarity from the closest type strains are shown in Table 14. There were 82 strains used for extract production and tested for antimicrobial activity. Some of the extracts, totally 58 extracts, had interesting antimicrobial activity with moderate and strong level. Some selected strains that can produce extracts with moderate and strong antimicrobial activity can be seen in Table 15. From 101 extracts that were tested for antiviral activity, only 46 extracts had promising antiviral activity with moderate (20-50% infectivity), strong (3-20% infectivity) and very strong level (< 3% infectivity). The nontoxic extracts that have strong antiviral activity against HCV are listed in Table 16.

Table 14. Some isolated Actinobacteria with low percentage similarity from the closest species based on 16S rRNA gene

No.	Strain	Closest type strain	Similarity (%)	Completeness of the sequence (%)
1	9BLSSO	*Streptomyces lanatus*	97.03	99.9
2	195336CR	*Mycobacterium palauense*	98.47	100
3	194938CR	*Kibdelosporangium banguiense*	98.74	100
4	195105	*Streptomyces cyaneus*	98.77	50.7
5	195107	*Streptomyces cyaneus*	98.77	50.6
6	195227GnCR	*Streptomyces filipinensis*	98.81	58.0
7	DHE 9-4	*Streptomyces spongiae*	98.83	100
8	196526CR	*Amycolatopsis thermalba*	98.85	100
9	190122BCR	*Streptomyces roietensis*	98.89	58.2
10	190233	*Streptomyces glomeratus*	98.96	33.3
11	SHP 1-2	*Streptomyces viridochromogenes*	99.03	99.9

Table 15. Selected strains that can produce extracts with moderate and strong activity against some microbes

No.	Strain	Production medium	Antimicrobial activity
1	SHP 1-2 (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Bacillus subtilis* (strong) *Staphylococcus aureus* (moderate) *Micrococcus luteus* (strong)
2	195105 (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Bacillus subtilis* (strong) *Staphylococcus aureus* (moderate) *Micrococcus luteu*s (moderate) *Mycobacterium smegmatis* (moderate) **Fungi** *Mucor hiemalis* (moderate)
3	195107 (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Bacillus subtilis* (strong) *Staphylococcus aureus* (strong) *Micrococcus luteu*s (moderate) *Mycobacterium smegmatis* (moderate) **Fungi** *Mucor hiemalis* (moderate)
4	196526CR (*Amycolatopsis* sp.)	5294	**Gram-positive bacteria** *Bacillus subtilis* (moderate) *Staphylococcus aureus* (moderate) **Fungi** *Mucor hiemalis* (moderate) *Candida albicans* (moderate)
5	194938CR (*Kibdelosporangium* sp.)	5254	**Gram-negative bacteria** *Escherichia coli* TolC (moderate) **Gram-positive bacteria** *Staphylococcus aureus* (moderate)
6	9BLSSO (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Staphylococcus aureus* (moderate) **Fungi** *Mucor hiemalis* (moderate)
7	195227GnCR (*Streptomyces* sp.)	5254	**Fungi** *Mucor hiemalis* (moderate)
8	190233 (*Streptomyces* sp.)	5294	**Fungi** *Mucor hiemalis* (moderate)

Table 16. List of non-toxic extracts having antiviral activity with strong level against HCV

No.	Strain	Extract
1	DHE 2-1	DHE 2-1_5254
2	DHE 2-1	DHE 2-1_5294
3	MAE 1-11	MAE 1-11_5294
4	MAE 1-11	MAE 1-11_SYP
5	SHP 1-4	SHP 1-4_5294
6	SHP 2-2	SHP 2-2_5294
7	SHP 6-6	SHP 6-6_5294
8	190231	190231_5294
9	C190221	C190221_5254
10	C194911	C194911_5294
11	C195321A	C195321A_5254
12	C195321A	C195321A_5294
13	C196921	C19692 _5254

3.2 Polyphasic taxonomy of *Streptomyces sp.* SHP 1-2

Morphology and melanin production

Streptomyces sp. SHP 1-2 grew well on GYM, ISP 2, ISP 3, ISP5, ISP6, ISP7, SSM+T and SSM-T medium agar and moderately on ISP 4 medium agar (Table 17). The morphology of the strain in GYM and ISP 2 medium can be seen in Figure 6. Scanning electron microscopy, after 14 days of growth on ISP 3, showed spiral chains of smooth surface spores (Figure 7).

Table 17. Growth and characteristics of *Streptomyces sp.* SHP 1-2 cultivated on various agar media after incubation for 14 days at 30 °C

Agar medium	Growth	Substrate mycelium color	Aerial mycelium color	Soluble pigment
Yeast extract-malt extract (ISP 2)	Good	Olive brown, ochre yellow	Traffic grey B, signal white	None
Oatmeal (ISP 3)	Good	Ivory	Telegrey 2, signal white	None
Inorganic salt-starch (ISP 4)	Moderate	Ivory	Telegrey 2, signal white	None
Glycerol-asparagine (ISP 5)	Good	Ivory, light ivory	Telegrey 2, signal white	None
Peptone-yeast extract-iron (ISP 6)	Good	Maize yellow	Signal white	None

Tyrosine (ISP 7)	Good	Ivory light, ivory	Traffic grey A, signal white	None
Synthetically Suter medium with tyrosine (SSM+T)	Good	Ocker brown, ivory	Signal white	None
Synthetically Suter medium without tyrosine (SSM-T)	Good	Ocher brown, ivory	Signal white	None

Figure 6. Morphology of *Streptomyces sp.* SHP 1-2 on GYM (left) and ISP 2 medium (right).

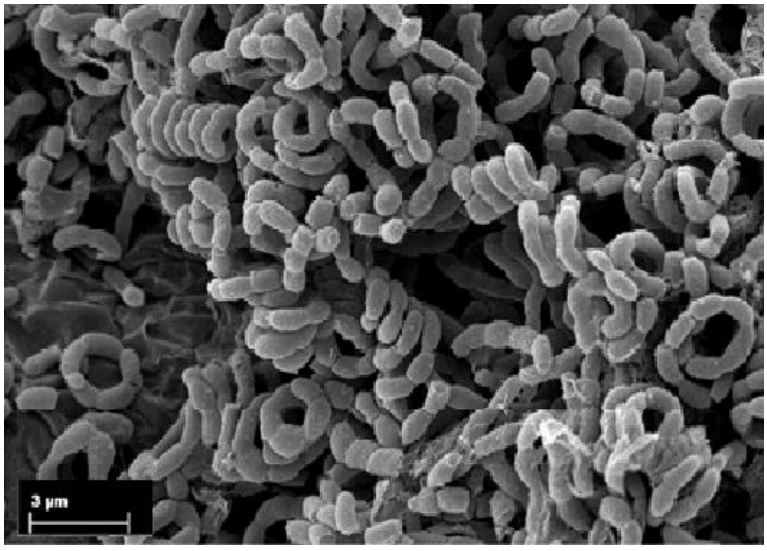


Figure 7. Scanning electron micrographs of aerial mycelium and spores of *Streptomyces sp.* SHP 1-2 after incubation on ISP 3 agar for 14 days at 30 °C.

3.1.1 Chemotaxonomy

The cell wall of *Streptomyces sp.* SHP 1-2 comprised of LL-diaminopimelic acid. The whole-cell sugar analysis of *Streptomyces sp.* SHP 1-2 suggested that the strain

contained glucose and xylose. The TLC chromatogram for amino acid of the cell wall and whole-cell sugar analysis can be seen in (Figure 8). The predominant fatty acids of *Streptomyces sp.* SHP 1-2 were anteiso-C15:0 (29.99%), iso-C16:0 (14.61%), anteiso-C17:0 (11.55%), iso-C15:0 (11.16%) and C16:0 (9.24%) (Figure 9). Its major menaquinones comprised as MK-9(H4) 9.31%, MK-9(H6) 68.03%, MK-9(H8) 22.65% in a ratio of 1:7:2 (Figure 10). The polar lipids were detected as diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and five unidentified polar lipids (Figure 11).

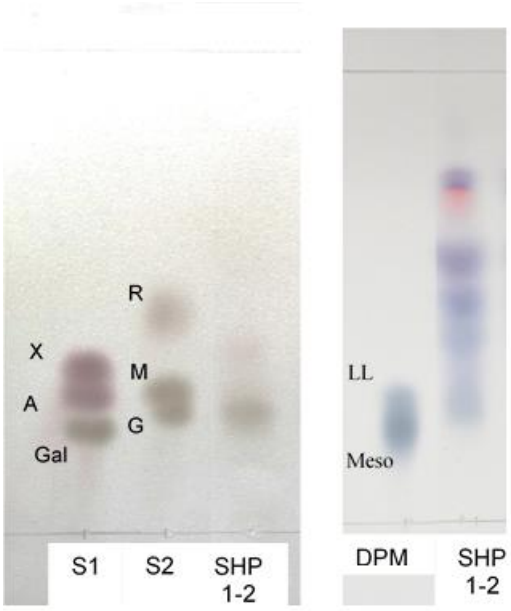
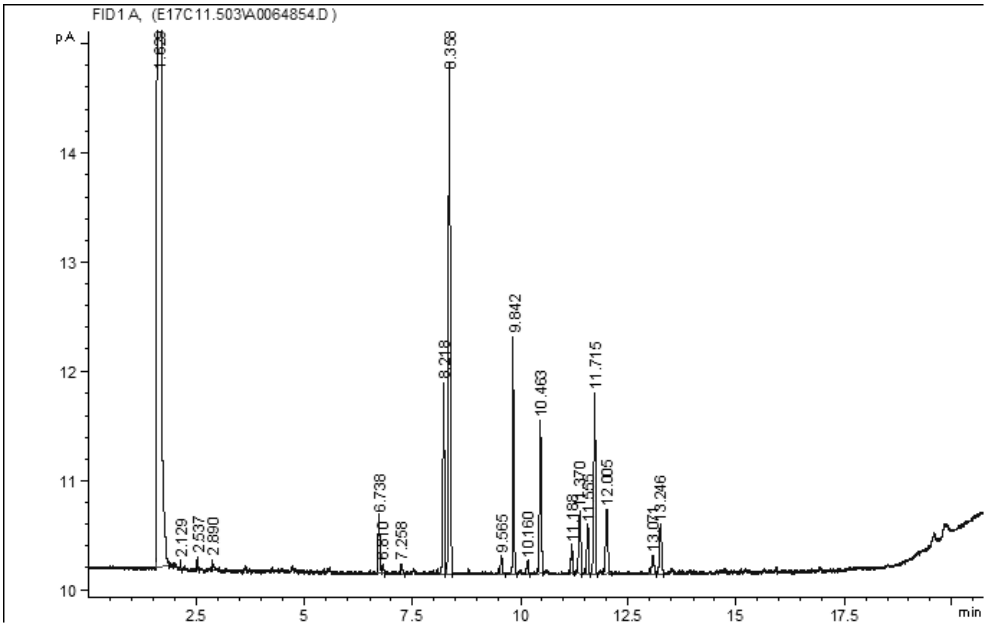


Figure 8. TLC chromatogram of whole-cell sugar analysis (left) and analysis amino acid of the cell wall (right) of *Streptomyces* sp. SHP 1-2. TLC chromatogram of whole-cell sugar analysis (left) and analysis amino acid of the cell wall (right) of Streptomyces sp. SHP 1-2. S1: Standard 1; S2: Standard 2; X: Xylose; A: Arabinose; Gal: Galactose; DPM: Diaminopimelic acid standard; LL: LL- Diaminopimelic acid; Meso: meso-Diaminopimelic acid; SHP 1-2: *Streptomyces* sp. SHP 1-2.

3.2.1 16S rRNA gene analysis

The almost complete 16S rRNA gene sequence of *Streptomyces* sp. SHP 1-2 (1,509 nucleotides) was determined and deposited under the GenBank accession number MK287949. Based on the result from EzTaxon server (http://www.ezbiocloud.net/taxonomy)[156], the strain was known to be closely related to the type strains of *Streptomyces viridochromogenes* NBRC 3113^T (99.03%), *Streptomyces malachitofuscus* NBRC 13059^T (99.03%), and *Streptomyces misionensis* DSM 40306^T (98.96%). *Streptomyces* sp. SHP 1-2 was also found to form a distinct phyletic line

from other 18 close species in the phylogenetic tree based on the 16S rRNA gene sequence (Figure 12).



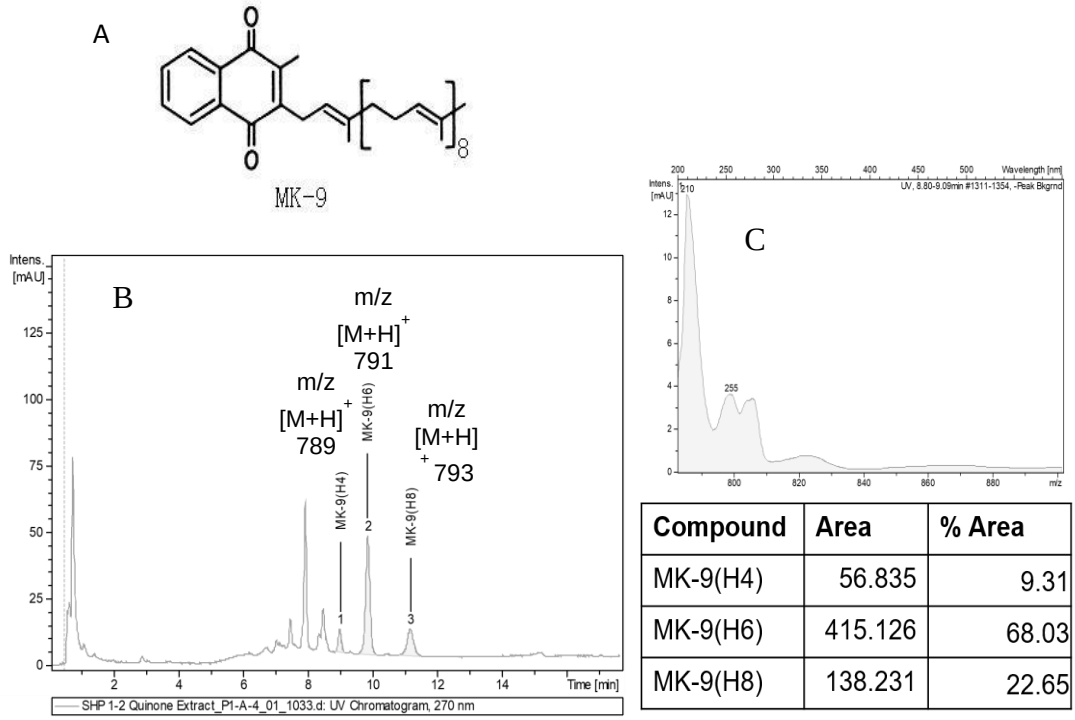
RT	Response	Ar/H	RFact	ECL	Peak Name	Percent
1.629	3.712E+8	0.026	----	7.011	SOLVENT PEAK	----
2.129	255	0.024	----	8.003		----
2.537	297	0.020	----	8.815		----
2.890	306	0.023	1.194	9.515	unknown 9.521	0.50
6.738	2374	0.036	0.986	13.618	14:0 ISO	3.17
6.810	364	0.033	----	13.671		----
7.258	389	0.033	0.976	13.998	14:0	0.51
8.218	**8561**	**0.039**	**0.962**	**14.622**	**15:0 ISO**	**11.16**
8.358	**23054**	**0.039**	**0.960**	**14.713**	**15:0 ANTEISO**	**29.99**
9.565	832	0.041	0.948	15.459	16:1 ISO H	1.07
9.842	**11404**	**0.042**	**0.946**	**15.625**	**16:0 ISO**	**14.61**
10.160	641	0.041	0.943	15.816	16:1 CIS 9	0.82
10.463	**7248**	**0.040**	**0.941**	**15.998**	**16:0**	**9.24**
11.188	1392	0.040	0.937	16.417	16:0 9? METHYL	1.77
11.370	3079	0.042	0.937	16.522	17:1 ANTEISO C	3.91
11.555	2388	0.042	0.936	16.629	17:0 ISO	3.03
11.715	**9119**	**0.042**	**0.935**	**16.722**	**17:0 ANTEISO**	**11.55**
12.005	3258	0.046	0.934	16.889	17:0 CYCLO	4.12
13.071	937	0.040	0.932	17.497	UNKNOWN 17.493 SM	1.18
13.246	2677	0.046	0.932	17.596	UNKNOWN 17.595 SM	3.38

Figure 9. GC chromatogram of fatty acid analysis of *Streptomyces* sp. SHP 1-2.

3.2.2 Multilocus sequence analysis (MLSA)

Based on MLSA analysis resulting from the concatenation of 5 house-keeping genes head to tail, it was determined that *Streptomyces* sp. SHP 1-2 and *Streptomyces fumigatiscleroticus* NRRL B-3856 ^{T} were very closely related with high significant bootstrap values or by the stability of the relationship when different phylogenetic algorithms are used, such as maximum-likelihood and neighbour-joining analyses (Figure 13). The MLSA distance between *Streptomyces* sp. SHP 1-2 and *S.*

fumigatiscleroticus NRRL B-3856^T was 0.058 (Table 18). *Streptomyces* sp. SHP 1-2 showed to have MLSA distance more than 0.007 with all of the other related species. The similarities of 16S rRNA gene sequence between *Streptomyces* sp. SHP 1-2 and *S. fumigatiscleroticus* NRRL B-3856 ^{T} was 97.72%.



Compound	Area	% Area
MK-9(H4)	56.835	9.31
MK-9(H6)	415.126	68.03
MK-9(H8)	138.231	22.65

Figure 10. Menaquinones detected in *Streptomyces* sp. SHP 1-2. A: structure of menaquinone-9 (MK-9); B: Chromatogram from LC-MS of detected menaquinones; C: UV Spectrum of menaquinone.

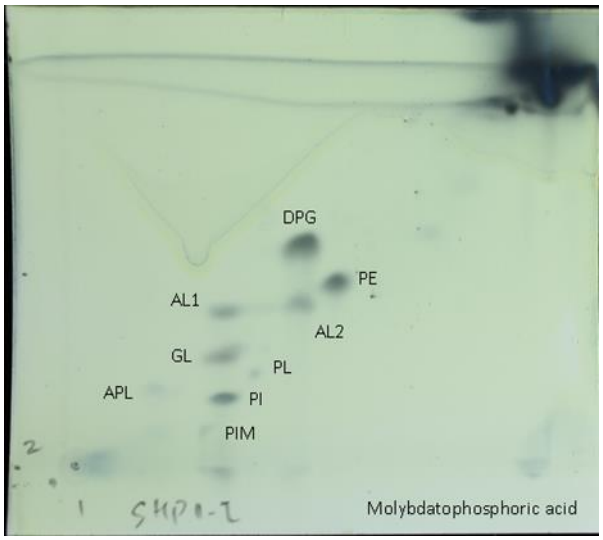


Figure 11. Polar lipid observed in *Streptomyces* sp. SHP 1-2. DPG: diphosphatidylglycerol; *PE: phosphatidethanolamine;* PI: phosphatidylinositol; *PIM: p*hosphatidylinositol mannoside; GL: unknown glycolipid; PL: unknown phospholipid; *A*PL: unknown aminophospholipid; AL1-2: unknown aminolipids.

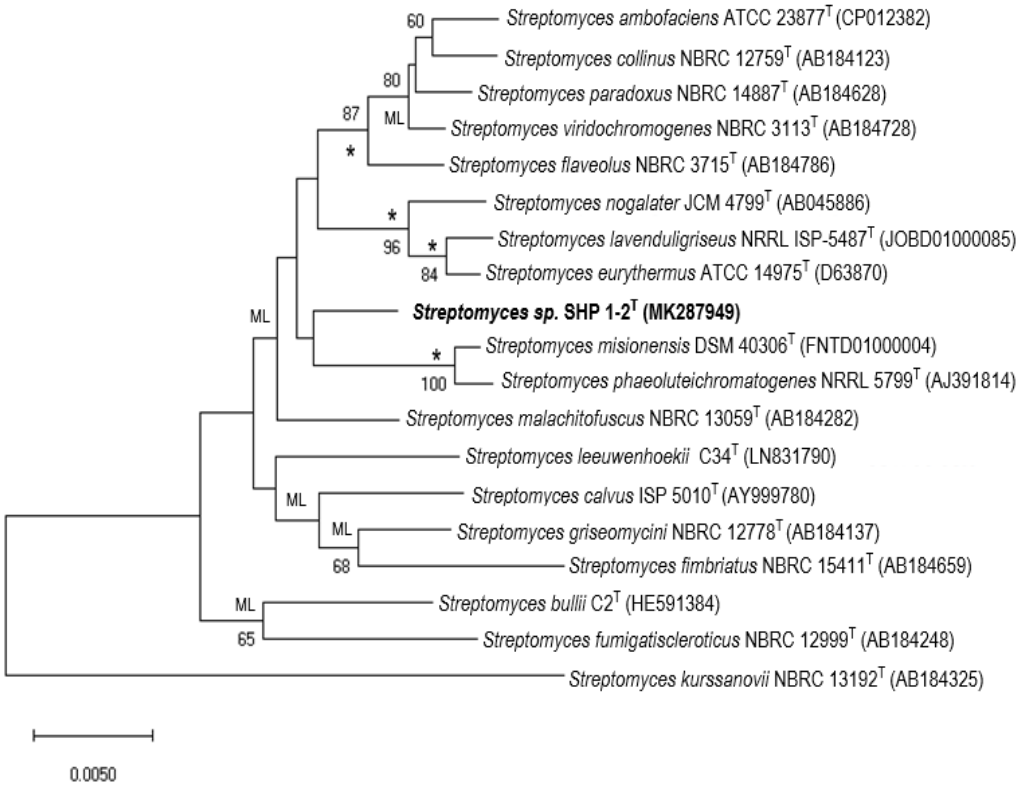


Figure 12. Neighbour-joining tree based on 16S rRNA gene sequences (1435 positions in the final dataset) showing relationships between strain SHP 1-2^T and the type strains of closely related *Streptomyces* species. The evolutionary distances were determined using the Tamura-Nei method[178]. Asterisks indicate branches of the tree that were also found using the maximum-likelihood and maximum-parsimony tree-making algorithms. ML specify nodes that were also recovered using the maximum-likelihood. Numbers at the nodes are percentage bootstrap values based on a neighbour-joining analysis of 1,000 replicates, only values above 50 % are shown. Bar 0.0050 substitutions per nucleotide position.

3.2.3 G+C content and full genome sequencing

The G+C content of *Streptomyces* sp. SHP 1-2 was found to be 73.37% based on HPLC analysis (Figure 14). Full genome analysis based on next-generation sequencing technique (NGS) exhibited that the genome size of the strain was 7,562765 bp.

3.2.4 Physiological and biochemical characteristic

Streptomyces sp. SHP 1-2 could grow at 15-37^{o}C (optimum 25-30^{o}C) and pH 6-9 (optimum pH 7). The strain was also able to grow in the presence of 7.5% NaCl, albeit the aerial mycelium was found only until 5% NaCl. Strain SHP 1-2 was sensitive to erythromycin (15 µg/disc), gentamycin 30 (µg/disc), tetracycline (30 µg/disc), vancomycin (30 µg/disc), and rifampicin (5 µg/disc). However it is resistant to ampicillin (10 µg/disc), cefotaxime (30 µg/disc), and penicillin G (6 µg/disc).

Streptomyces sp. SHP 1-2 was only able to use glucose as their source of carbon. The results from API ZYM suggested that the strain possessed strong enzymatic activities for phosphatase alkaline, leucine arylamidase, alpha-glucosidase, and N-acetyl-beta-glucoseamidase; however, it had weak activity for esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cysteine arylamidase, phosphatase acid, naphtol-AS-BI-phosphohydrolase, beta-galactosidase, and beta-glucosidase. From API Coryne tests, it was found that the strain had positive results for alkaline phosphatase, alpha-glucosidase, N-acetyl-beta-glucoseamidase, esculin (beta-glucosidase), and gelatinase (Table 19).

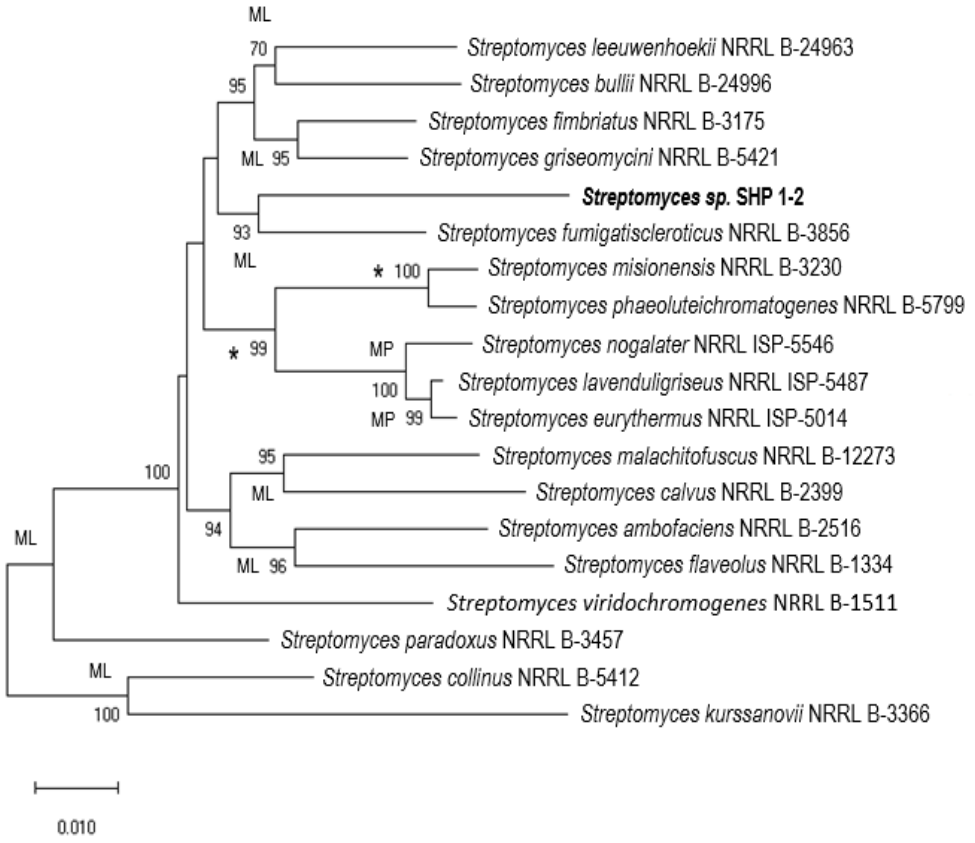
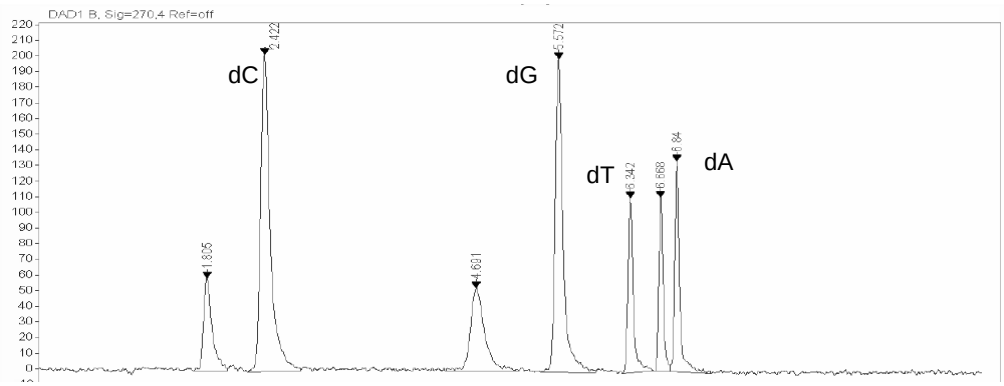


Figure 13. Neighbour-joining tree based on concatenated partial sequences of the house-keeping genes *atpD*, *gyrB*, *recA*, *rpoB* and *trpB*. The evolutionary distances were determined using the Tamura-Nei method[178]. There were totally 2528 positions in the final dataset. Asterisks indicate branches of the tree that were also found using the maximum-likelihood and maximum-parsimony tree-making algorithms. ML and MP specify nodes that were also recovered using the maximum-likelihood and maximum-parsimony, respectively. Numbers at the nodes are percentage bootstrap values based on a neighbour-joining analysis of 1,000 replicates, only values above 50 % are shown. Bar 0.010 substitutions per nucleotide position.

Table 18. MLSA distance between *Streptomyces* sp. SHP 1-2 and other related strains

Compared strain	MLSA distance
Streptomyces misionensis NRRL B-3230	0.072
Streptomyces paradoxus NRRL B-3457	0.094
Streptomyces viridochromogenes NRRL B-1511	0.081
Streptomyces lavenduligriseus NRRL ISP-5487	0.073
Streptomyces malachitofuscus NRRL B-12273	0.083
Streptomyces eurythermus NRRL ISP-5014	0.075
Streptomyces phaeoluteichromatogenes NRRL B-5799	0.073
Streptomyces nogalater NRRL ISP-5546	0.078
Streptomyces calvus NRRL B-2399	0.079
Streptomyces flaveolus NRRL B-1334	0.085
Streptomyces leeuwenhoekii NRRL B-24963	0.072
Streptomyces fumigatiscleroticus NRRL B-3856	0.058
Streptomyces griseomycini NRRL B-5421	0.069



Compound	Area	% Area	%GC
Deoxycytidine (dC)	1460.980	40.363	**73.37**
Deoxyguanosine (dG)	1194.580	33.003	
Deoxythymidine (dT)	452.515	12.502	
Deoxyadenosine (dA)	511.538	14.132	

Figure 14. G+C content from the genome of *Streptomyces* sp. SHP 1-2 based on HPLC anlaysis.

Table 19. API ZYM and API Coryne test result for *Streptomyces* sp. SHP 1-2

API ZYM	Activity	API Coryne	Activity
Alkaline phosphatase	++	Nitrate reduction	-
Butyrate esterase (C4)	(+)	Pyrazinamidase	-
Caprylate esterase lipase (C8)	(+)	Pyrrolidony arylamidase	-
Myristate lipase (C14)	(+)	Alkaline phosphatase	+
Leucine arylamidase	++	β-glucuronidase	-
Valine arylamidase	(+)	β-galactosidase	-
Cystine arylamidase	(+)	α-Glucosidase	+
Trypsin	-	N-acetyl-β-glucosamidase	+
α-Chymotrypsin	-	Esculin (β-glucosidase)	+
Acid phosphatase	(+)	Urease	-
Naphtol-AS-BI-phosphohydrolase	(+)	Gelatine (hydrolysis)	+
α-Galactosidase	-	Glucose fermantation	-
β-Galactosidase	(+)	Ribose fermentation	-
β-Glucoronidase	-	Xylose fermentation	-
α-Glucosidase	++	Mannitol fermentation	-
β-Glucosidase	(+)	Maltose fermentation	-
N-acetyl-β-glucosaminidase	++	Lactose fermentation	-
α-Mannosidase	-	Sucrose fermentation	-
α-Fucosidase	-	Glycogen fermentation	-

++ more positive result; + positive result; (+) weakly positive result; - negative result.

3.2.5 Phenotypic characteristic between *Streptomyces* sp. SHP 1-2 and its closest related strains

To make more support for separation between species, some phenotypic properties of *Streptomyces* sp. SHP 1-2 was compared to the very close strains based on its 16S rDNA and MLSA result. *Streptomyces malachitofuscus* DSM 40332^{T}, *Streptomyces viridochromogenes* DSM 40110^{T} and *Streptomyces misionensis* DSM 40306^{T} were used as they are very close based on their 16S rDNA. *Streptomyces fumigatiscleroticus* DSM 43154^{T} was employed because it is very close according to MLSA result (Table 20).

Table 20. Some phenotypic comparison between *Streptomyces* sp. SHP 1-2 and its closest related strains

Characteristic	1	2	3	4	5
ISP 2 - aerial mycelium	Traffic grey B, signal white	Sparse	Grey	Blue grey	None
ISP 6 - soluble pigment	None	None	Brown	Brown	None
ISP 7 - soluble pigment	None	Red	Black	None	None
Use of carbohydrate					
Glucose	+	-	+	+	+
Arabinose	-	+	+	+	(+)
Sucrose	-	-	+	-	-
Xylose	-	-	-	+	++
Inositol	-	-	+	+	(+)
Mannitol	-	+	(+)	+	(+)
Fructose	-	+	+	+	(+)
Rhamnose	-	+	+	-	-
Raffinose	-	+	-	-	-
API ZYM					
Phosphatase alcaline	++	++	++	++	++
Trypsin	-	(+)	+	+	-
Chymotrypsin	-	(+)	+	(+)	(+)
α-Glucosidase	++	+	++	-	++
N-acetyl-beta-glucoseamidase	++	-	+	++	++
α-Mannosidase	-	++	++	++	++
API Coryne					
Nitrate reduction	-	+	-	-	-
Pyrrolidonyl arylamidase	-	-	-	-	+
β-Galactosidase	-	+	-	-	+
Esculin (beta glucosidase)	+	+	-	-	+
Gelatine(hydrolysis)	+	+	+	-	+

++ more positive result; + positive result; - negative result; (+) weakly positive result; 1: Strain SHP 1-2; *2: S. fumigatiscleroticus* DSM 43154 T; 3: *S. malachitofuscus* DSM 40332; 4: *S. viridochromogenes* DSM 40110; 5: *S. misionensis* DSM 40306.

3.3 Compounds isolated from *Streptomyces* sp. SHP 1-2 and their biological activity

This experiment was conducted together with Gian Primahana (Ph.D student in the working group "Microbial drugs" of the HZI, Braunschweig, Germany), Dr. Rizna T. Dewi (scientist from Research Center of Chemistry, LIPI, Indonesia), and Dr. Enge Sudarman (scientist in the working group "Microbial drugs" of the HZI, Braunschweig, Germany). There were six compounds isolated from *Streptomyces* sp. SHP 1-2. Two of them were new compounds (indolactam variant 1 and indolactam variant 2). Four known compounds were Lyngbyatoxin A, Cyclo(phenylalanylprolyl), Indolactam V, and Piericidin A1 (Figure 15).

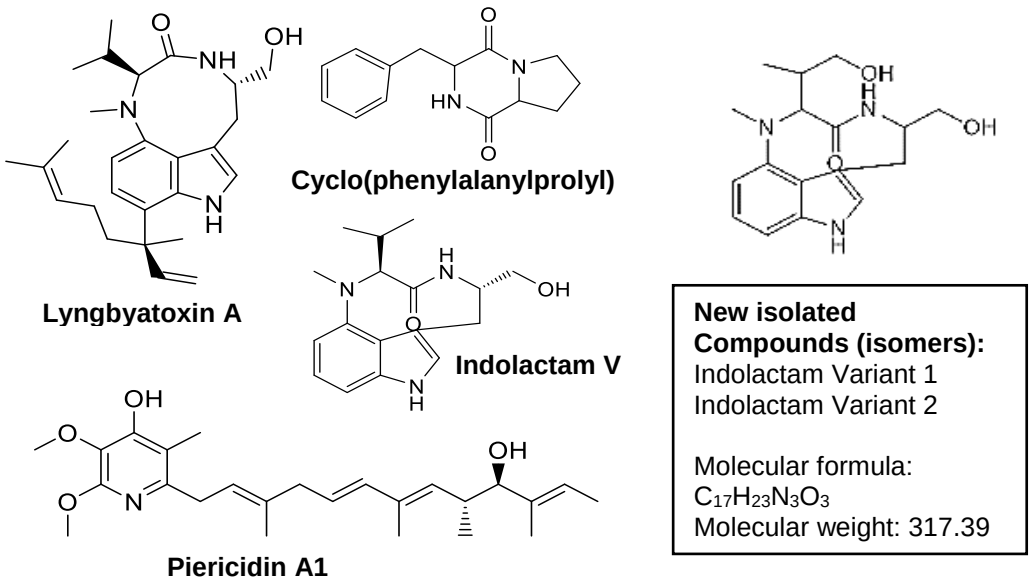


Figure 15. Compounds isolated from *Streptomyces* sp. SHP 1-2.

Piericidin A1 showed potent antimicrobial activity against *Escherichia coli* TolC, *Chromobacterium violaceum*, *Bacillus subtilis*, and *Micrococcus luteus* with the MIC values less than 5 µg/ml. Piericidin A1 also had activity in the inhibition of almost all tested mammalian cells. Besides, piericidin A1 was the only isolated compound that possessed antinematode activity. Indolactam variant 1 and indolactam variant 2 showed no activity against all of the tested bacteria, fungi, and nematode. Indolactam variant 1 inhibited moderately the growth of KB-3-1 and PC-3 cells with IC_{50} 8 and 31 µg/ml, respectively. Indolactam variant 2 could inhibit reasonably KB-3-1, PC-3, and SK-OV-3 cell lines with the IC_{50} 3.5, 18, and 25 µg/ml respectively. Lyngbyatoxin A showed antimicrobial activity against some Gram-positive bacteria *Staphylococcus aureus*, *B. subtilis*, and *M. luteus* with the MIC less than 5 µg/ml. Lyngbyatoxin A

exhibited cytotoxic activity against all of eight mammalian cells; however, no activity was observed against nematode *Caenorhabditis elegans* (Table 21).

Table 21. Biological activity of compounds isolated from strain SHP 1-2.

Tested orgainsm	Compound					
	1	**2**	**3**	**4**	**5**	**6**
Microbes	**MIC (µg/ml)**					
Escherichia coli	> 66.7	ND	> 66.7	> 66.7	ND	> 66.7
Escherichia coli TolC	2.32	ND	> 66.7	> 66.7	ND	> 66.7
Chromobacterium violaceum	1.16	ND	> 66.7	> 66.7	ND	> 66.7
Pseudomonas aeruginosa	> 66.7	ND	> 66.7	> 66.7	ND	> 66.7
Staphylococcus aureus	> 66.7	ND	> 66.7	> 66.7	ND	4.75
Bacillus subtilis	2.5	ND	> 66.7	> 66.7	ND	2.38
Micrococcus luteus	4.65	ND	> 66.7	> 66.7	ND	4.75
Mycobacterium smegmatis	> 66.7	ND	> 66.7	> 66.7	ND	> 66.7
Mucor hiemalis	> 66.7	ND	> 66.7	> 66.7	ND	> 66.7
Pichia anomala	> 66.7	ND	> 66.7	> 66.7	ND	> 66.7
Candida albicans	> 66.7	ND	> 66.7	> 66.7	ND	> 66.7
Mamalian cells	**IC50 (µg/ml)**					
L-929	5.1	ND	> 100	> 100	ND	4.1
KB-3-1	0.57×10^{-3}	ND	8	3.5	ND	2
A-549	ND	ND	> 100	> 100	ND	0.45×10^{-3}
PC-3	0.33×10^{-3}	ND	31	18	ND	1.7
MCF-7	8.55×10^{-3}	ND	> 100	> 100	ND	3.4
A-431	17×10^{-3}	ND	> 100	> 100	ND	2.3
SK-OV-3	0.64×10^{-3}	ND	> 100	25	ND	5.5
HUVEC	25×10^{-3}	ND	ND	ND	ND	1.9
Nematode	**LC50 (µg/ml)**					
Caenorhabditis elegans	10.8	> 100	> 100	> 100	> 100	> 100

1: Piericidin A1; 2: Indolactam V; 3: Indolactam variant 1; 4: Indolactam variant 2; 5: Cyclo (phenylalanyalprolyl); 6: Lyngbyatoxin A; ND: Not Determined.

3.4 Taxonomy study and extract analysis of strain MAE 1-11

3.4.1 Morphology and melanin production

Growth of isolate MAE 1-11 was well observed on ISP 2, ISP 3, ISP 4, ISP 5, ISP 6, ISP 7, SSM+T and SSM-T medium. The strain can also produce melanin in ISP 6 and SSM+T medium (Table 22). The morphology of the isolate MAE 1-11 in GYM and ISP 2 medium can be seen in Figure 16.

Table 22. Growth and characteristics of strain MAE 1-11 grown on various agar media after incubation for 14 days at 30°C

Agar medium	Substrate mycelium color	Aerial mycelium color	Soluble pigment
Yeast extract-malt extract (ISP 2)	Maize yellow; Brown beige	Cream	None
Oatmeal (ISP 3)	Maize yellow; Saffron yellow	Grey white; Signal white	None
Inorganic salt- starch (ISP 4)	Sand yellow; Broom yellow	Signal white	None
Glycerol-asparagine (ISP 5)	Sand yellow; Lemon yellow	Signal white	None
Peptone-yeast extract-iron (ISP 6)	Yellow olive; olive drab; sepia brown	Signal white	Chocolate brown; mahogany brown
Tyrosine (ISP 7)	Brown beige; maize yellow	Silk grey; grey white; signal white	None
Synthetically Suter medium with tyrosine (SSM+T)	Olive brown; clay brown	None	Brown grey
Synthetically Suter medium without tyrosine (SSM-T)	Maize yellow; lemon yellow	None	None

3.4.2 16S rRNA gene analysis

The almost complete 16S rRNA gene (1,498 nt) was analyzed by EzTaxon server (http://www. ezbiocloud.net/taxonomy)[156] and the result suggested that isolate MAE 1-11 was found to be closely related to the type strains of *Kitasatospora albolonga* NBRC 13465(T) (99.93%), *Streptomyces cavourensis* NBRC 13026^T (99.86%), and *Streptomyces bacillaris* NBRC 13487^T (99.38%). In the phylogenetic tree based on the 16S rRNA gene sequences of strain MAE 1-11 and its closest type strains, the isolate MAE 1-11 formed a distinct subclade and clustered with *S. cavourensis* NBRC

13026^T, close to *K. albolonga* NBRC 13465^T and *Streptomyces araujoniae* ASBV-1^T by using Neighbour-Joining method[161]. However, the subclade consisting of strain MAE 1-11 and *S. cavourensis* NBRC 13026^T were not founded in the tree constructed by using the maximum-likelihood and maximum-parsimony algorithms (Figure 17).

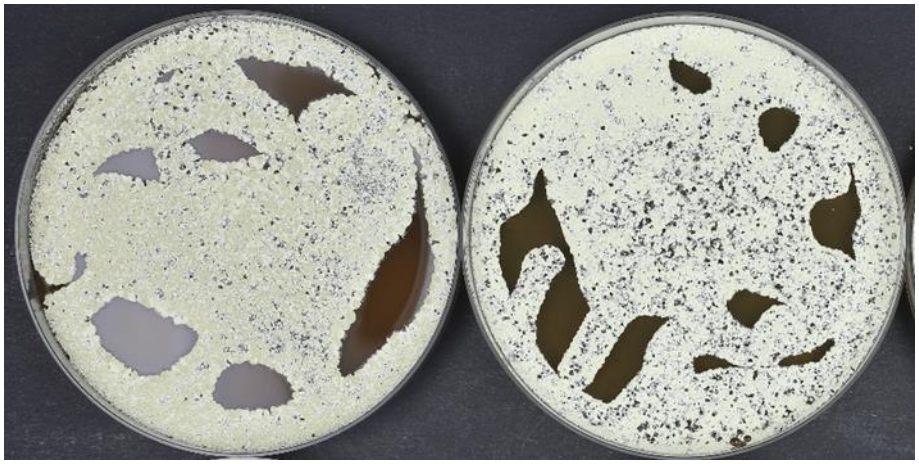


Figure 16. Morphology of strain MAE 1-11 on GYM (left) and ISP 2 medium (right).

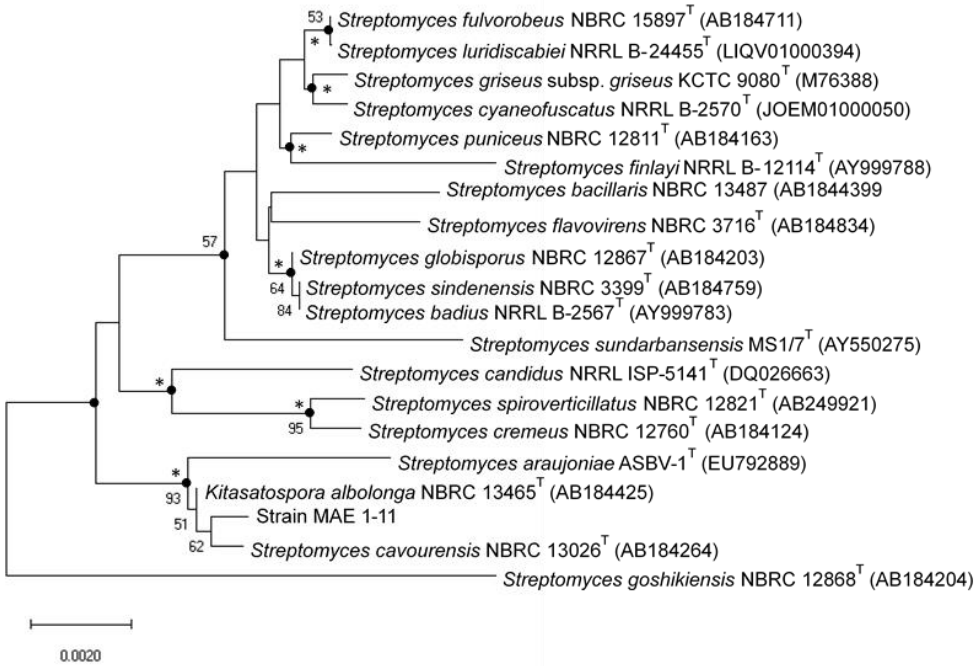


Figure 17. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences between strain MAE 1-11 and its closely related neighbours. The evolutionary history was inferred using the Neighbor-Joining method[161]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches[162]. The evolutionary distances were computed using the Kimura 2-parameter method[175]. There were a total of 1426 positions in the final dataset. Evolutionary analyses were conducted in MEGA X[158]. Asterisks and filled circles indicate branches of the tree that were also found using the maximum-likelihood and maximum-parsimony tree-making algorithms, respectively.

3.4.3 Physiological and biochemical characteristic

Strain MAE 1-11 was able to grow in the presence of 10% NaCl; however, the aerial mycelium was found only until 5% NaCl. The strain could be cultivated by using glucose, xylose, mannitol, and fructose (Table 23).

Table 23. Some physiology properties of strain MAE 1-11

Characteristic	Observation		Observation
Use of carbohydrate		Use of carbohydrate	
Glucose	+	Mannitol	+
Arabinose	-	Fructose	+
Sucrose	(+)	Rhamnose	(+)
Xylose	+	Raffinose	(+)
Inositol	(+)	Cellulose	-
NaCl tolerance	10%; aerial mycelium until 5%		
API ZYM		API ZYM	
Phosphatase alkaline	++	Naphtol-AS-BI-phosphohydrolase	+
Esterase (C4)	+	α-Galactosidase	-
Esterase Lipase (C8)	+	β-Galactosidase	(+)
Lipase (C14)	++	β-Glucuronidase	-
Leucin arylamidase	++	α-Glucosidase	++
Valine arylamidase	++	β-Glucosidase	++
Cystine arylamidase	+	N-acetyl-beta-glucoseamidase	-
Trypsin	++	α-Mannosidase	-
Chymotrypsin	++	α-Fucosidase	-
Phosphatase acid	+		
API Coryne		API Coryne	
Nitrate reduction	+	Gelatine (hydrolysis)	+
Pyraziamidase	-	Glucose fermentation	-
Pyrrolidonyl arylamidase	-	Ribose fermentation	-
Alkaline phosphatase	+	Xylose fermentation	-
β-Glucuronidase	-	Mannitol fermentation	-
β-Galactosidase	+	Maltose fermentation	-
α-Glucosidase	+	Lactose fermentation	-

N-acetyl -beta glucoseamidase	-	Sucrose fermentation	-
Esculin (beta glucosidase)	+	Glycogen fermentation	-
Urease	+		

++ more positive result; + positive result; (+) weakly positive result; - negative result.

3.4.4 Raw extract analysis

The raw extract derived from SYP medium was analyzed by LC-HRMS (MAXIS) and the chromatogram result can be seen in Figure 18. It was found that the peak in the retention time between 14.74-14.99 min (compound no.1) has the antiviral activity against HCV. From the mass analysis, it was known that the mass of the compound is 604.3978 Da. Using the data of the compound mass and the UV spectrum as well as submitting it in the Dictionary Natural Product, it was then figured out that compound no. 1 is Bafilomycin D. In addition, by comparing with the pure Bafilomycin D, it was also revealed that the compound no. 1 is Bafilomycin D (Figure 19).

3.5 Taxonomic study of strain 196526CR

3.5.1 Morphology and melanin production

Strain 196526CR could be grown well on ISP 2, ISP 5, ISP 6, ISP 7, SSM+T and SSM-T medium. Growth in ISP 3 was sparse and no growth was observed in ISP 4. The strain produced no melanin in the melanin production medium (Table 24). The morphology of the isolate 196526CR in GYM and ISP 2 medium can be seen in Figure 20.

3.5.2 16S rRNA gene analysis

The almost complete 16S rRNA gene (1,458 nt) was used in the EzTaxon analysis (http://www. ezbiocloud.net/taxonomy)[156]. It was found that strain 196526CR was close to the type strains of Amycolatopsis thermalba SF45T (98.85%), Amycolatopsis deserti GY024T (98.84%), Amycolatopsis methanolica 239T (98.75%) and Amycolatopsis endophytica KLBMP 1221T (98.75%). In a phylogenetic tree, the isolate 196526CR formed a distinct subclade and clustered with Amycolatopsis deserti GY024T, close to Amycolatopsis endophytica KLBMP 1221T and Amycolatopsis thermophile GY088T by using the neighbour-Joining method. The subclade consisting of strain 196526CR and Amycolatopsis deserti GY024T were also founded in the tree constructed maximum-parsimony algorithm (Figure 21).

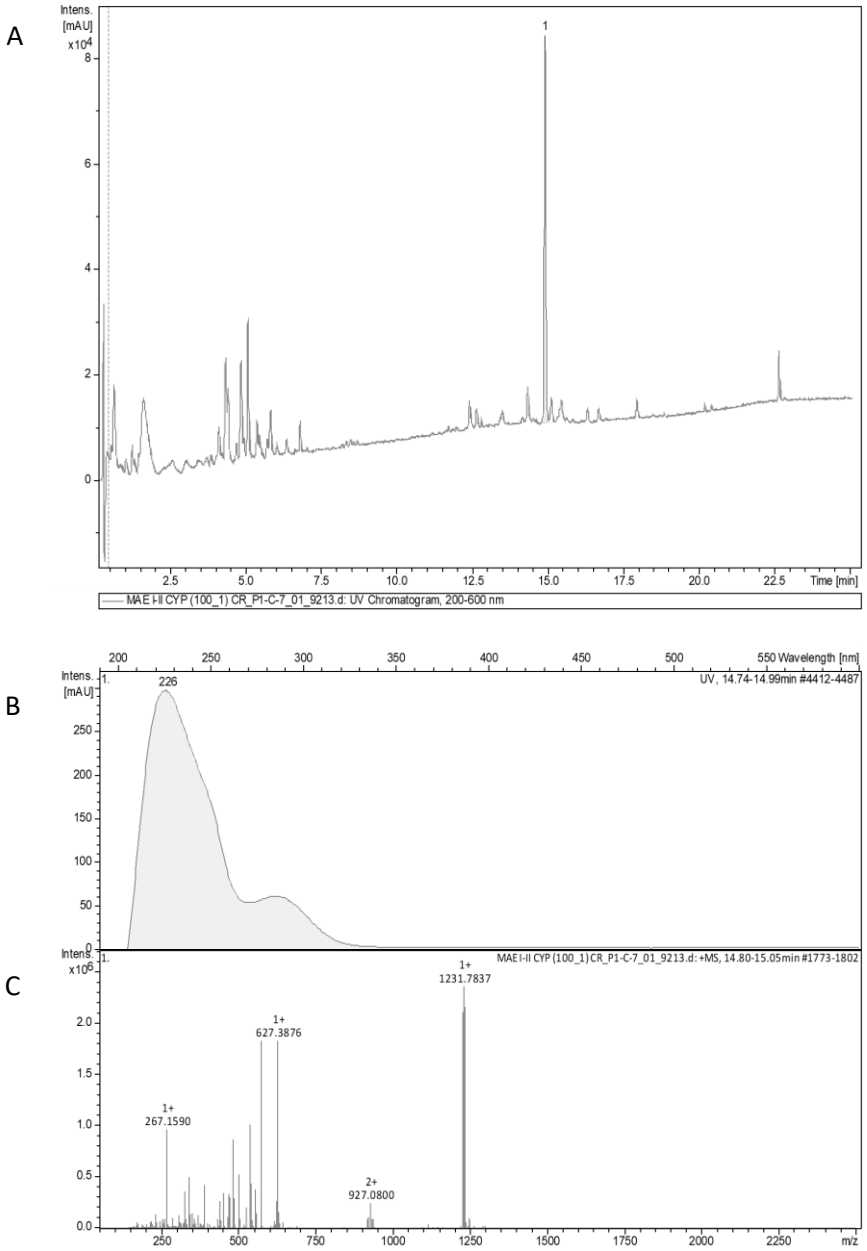


Figure 18. LC-HRMS analysis result of raw extract of strain MAE 1-11 produced by SYP medium. A: Chromatogram of raw extract detected by 200-600 nm; B: UV Spectrum of compound no. 1 from the chromatogram; C: Mass spectrum of compound no. 1 from the chromatogram.

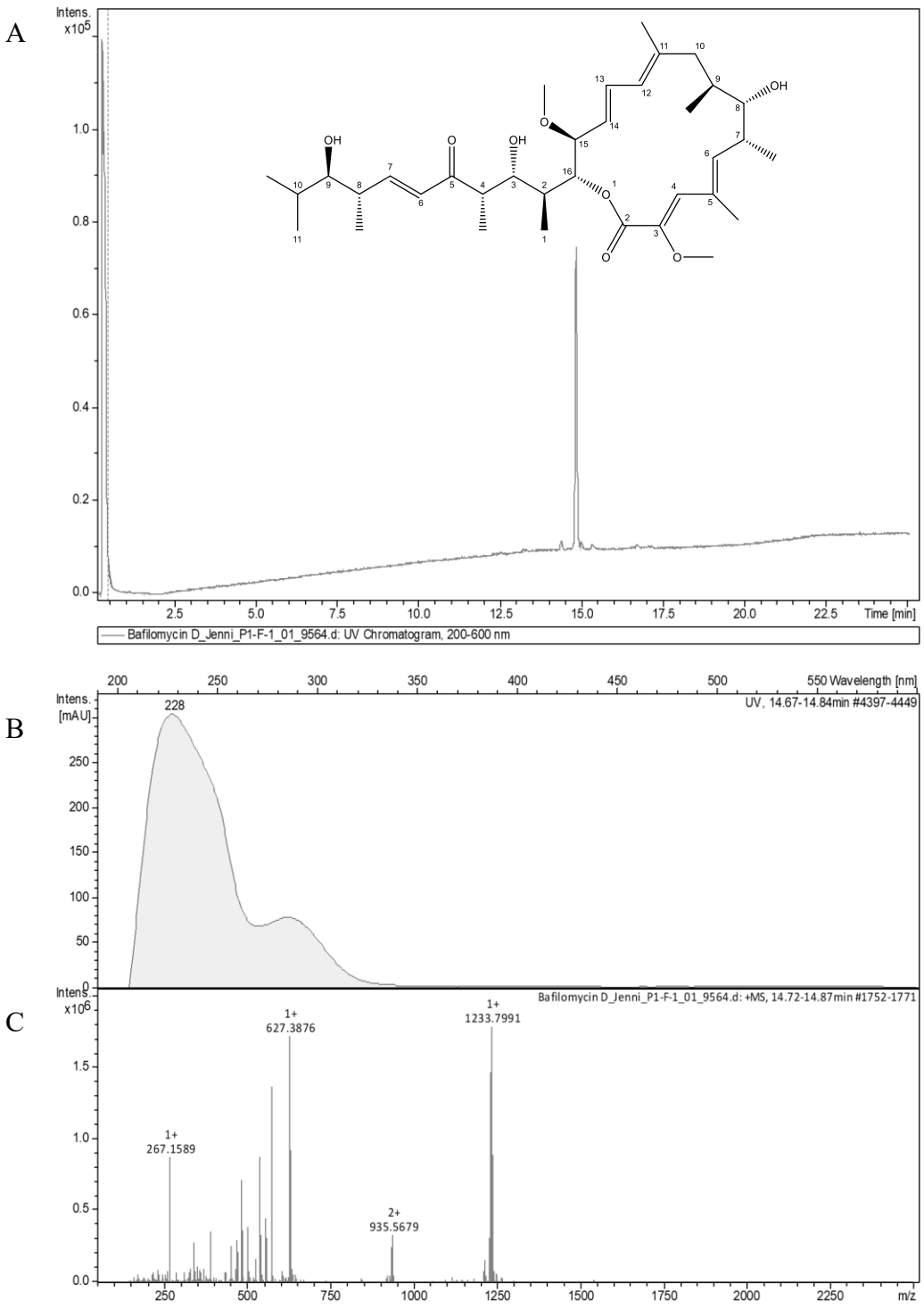


Figure 19. LC-HRMS analysis result of Bafilomycin D. A: Structure molecule of bafilomycin D and its chromatogram detected by 200-600 nm and the; B: UV Spectrum of bafilomycin D; C: Mass spectrum of bafilomycin D.

Table 24. Growth and characteristics of isolate 196526CR grown on various agar media after incubation for 14 days at 30°C

Agar medium	Growth	Substrate mycelium color	Aerial mycelium color	Soluble pigment
Yeast extract-malt extract (ISP 2)	Good	Signal yellow	Pure white	None
Oatmeal (ISP 3)	Sparse	Sand yellow	Cream	None
Inorganic salt-starch (ISP 4)	None	None	None	None
Glycerol-asparagine (ISP 5)	Good	Sand yellow	Pure white	None
Peptone-yeast extract-iron (ISP 6)	Good	Honey yellow	None	None
Tyrosine (ISP 7)	Good	Golden yellow	Traffic white	None
Synthetically Suter medium with tyrosine (SSM+T)	Good	Golden yellow	Cream	None
Synthetically Suter medium without tyrosine (SSM-T)	Good	Golden yellow	Light ivory	None

Figure 20. Morphology of strain 196526CR on GYM (left) and ISP 2 medium (right)

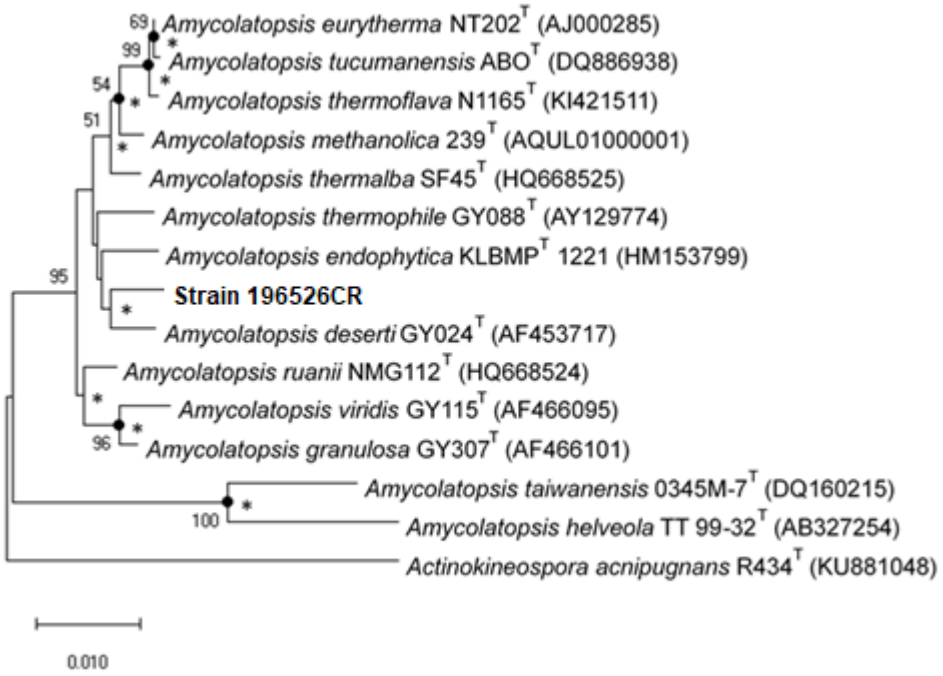


Figure 21. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences between strain 196526CR and its closely related neighbours. The evolutionary history was inferred using the Neighbor-Joining method[161]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches[162]. The evolutionary distances were computed using the Kimura 2-parameter method[175]. There were a total of 1344 positions in the final dataset. Evolutionary analyses were conducted in MEGA X[158]. Asterisks and filled circles indicate branches of the tree that were also found using the maximum-likelihood and maximum-parsimony tree-making algorithms, respctively.

3.5.3 Physiological and biochemical characteristic

Strain 196526CR grew in the presence of 5% NaCl. The strain could also grow well by using glucose and fructose. Weak growth was detected on the medium containing arabinose, xylose, mannitol, and rhamnose. The results from API ZYM suggested that the strain possessed substantial enzymatic activities for β-Glucosidase and N-acetyl-beta-glucoseamidase. It also had good activities for phosphatase alkaline, phosphatase acid, urease, and gelatinase (Table 25).

3.5.4 Secondary metabolites produced by strain 196526CR

In 5294HG-S medium, strain 196526CR produced coproporphyrin III, zinc coproporphyrin III, and nitrosoxacin C (Figure 22). Coproporphyrin III and zinc coproporphyrin III was detected both in the biomass and in the XAD extract, while nitrosoxacin C was found in the biomass extract (Figure 23 and Figure 24). It could

inhibit Bacillus substilis (MIC = 33.4 µg/ml) and Staphylococcus aureus (MIC = 33.4 µg/ml).

Table 25. Some physiology properties of strain 196526CR

Characteristic	Observation		Observation
Use of carbohydrate		**Use of carbohydrate**	
Glucose	+	Mannitol	(+)
Arabinose	(+)	Fructose	+
Sucrose	-	Rhamnose	(+)
Xylose	(+)	Raffinose	-
Inositol	-	Cellulose	-
NaCl tolerance	5%		
API ZYM		**API ZYM**	
Phosphatase alkaline	+	Naphtol-AS-BI-phosphohydrolase	-
Esterase (C4)	-	α-Galactosidase	-
Esterase Lipase (C8)	(+)	β-Galactosidase	(+)
Lipase (C14)	(+)	β-Glucuronidase	-
Leucin arylamidase	++	α-Glucosidase	-
Valine arylamidase	++	β-Glucosidase	++
Cystine arylamidase	(+)	N-acetyl-beta-glucoseamidase	++
Trypsin	(+)	α-Mannosidase	(+)
Chymotrypsin	-	α-Fucosidase	-
Phosphatase acid	+		
API Coryne		**API Coryne**	
Nitrate reduction	-	Gelatine(hydrolysis)	+
Pyraziamidase	-	Glucose fermentation	-
Pyrrolidonyl arylamidase	-	Ribose fermentation	-
Alkaline phosphatase	+	Xylose fermentation	-
β-Glucuronidase	-	Mannitol fermentation	-
β-Galactosidase	-	Maltose fermentation	-
α-Glucosidase	-	Lactose fermentation	-
N-acetyl -beta glucoseamidase	+	Sucrose fermentation	-
Esculin (beta glucosidase)	+	Glycogen fermentation	-
Urease	+		

++ more positive result; + positive result; - negative result; (+) weakly positive result

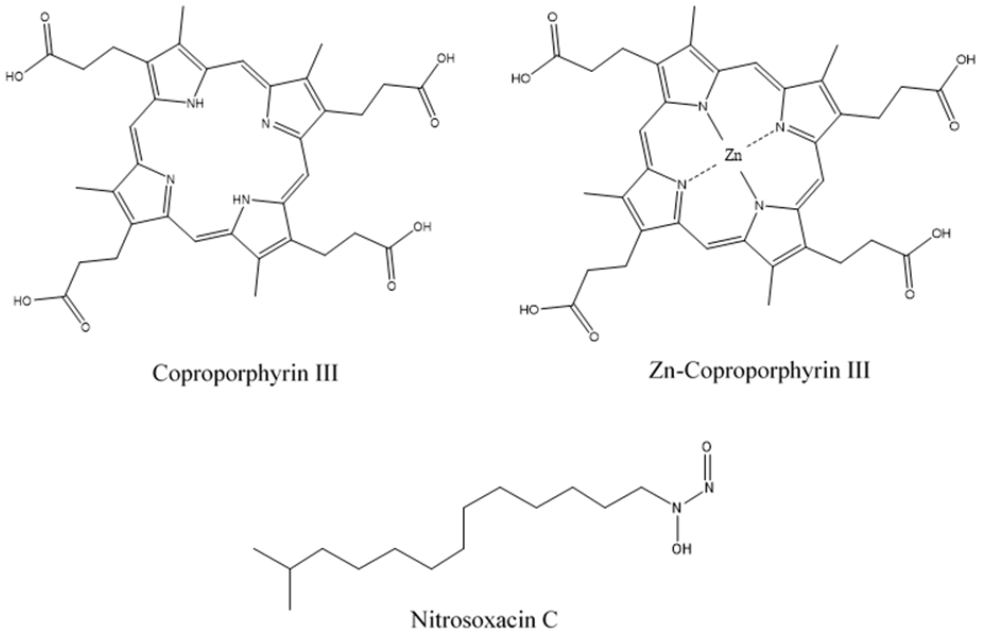


Figure 22. Structure of secondary metabolites produced by by strain 196526CR.

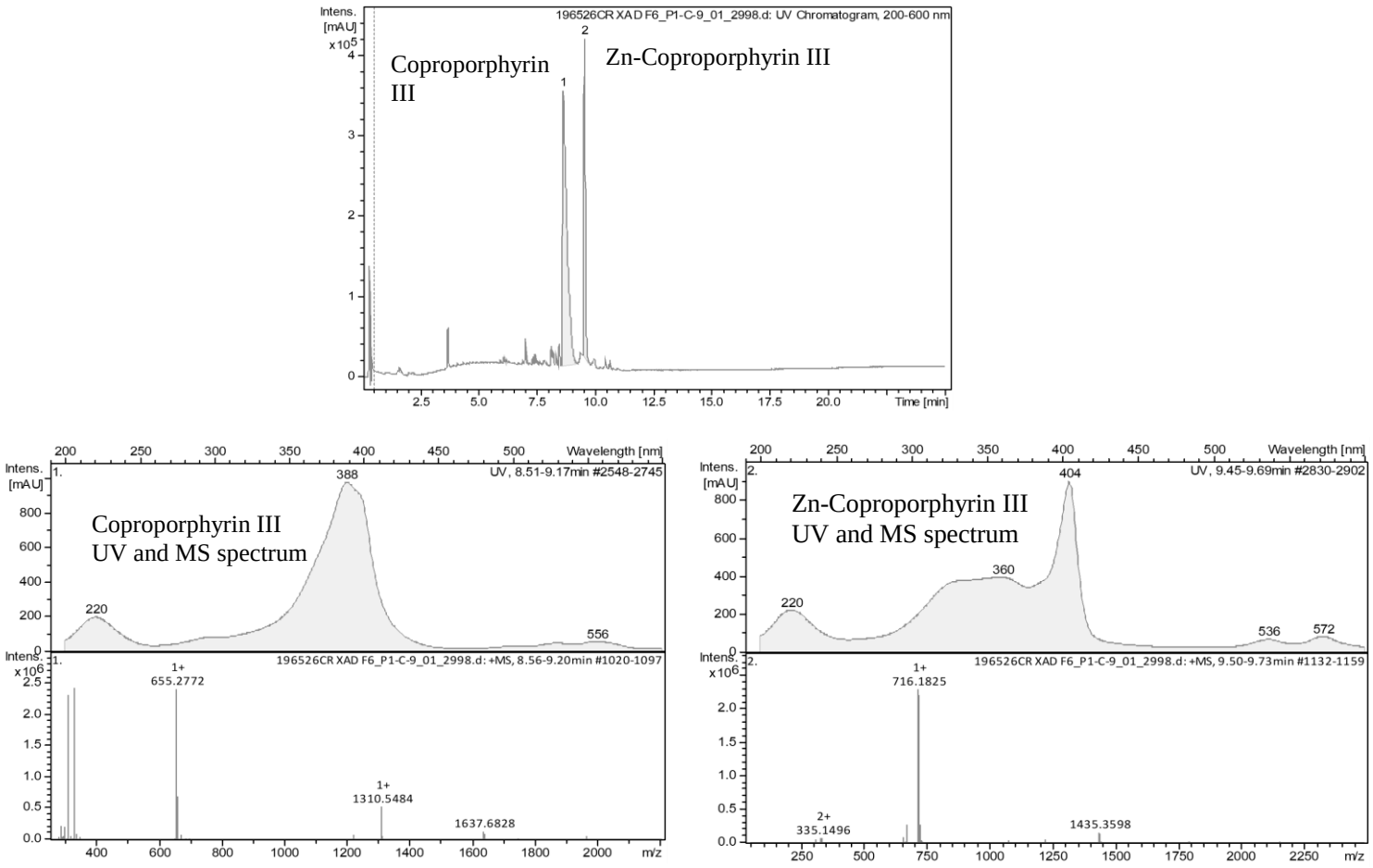


Figure 23. Chromatogram, UV and MS spectrum of Coproporphyrin III and Zn- Coproporphyrin III found in the XAD extract of strain 196526CR.

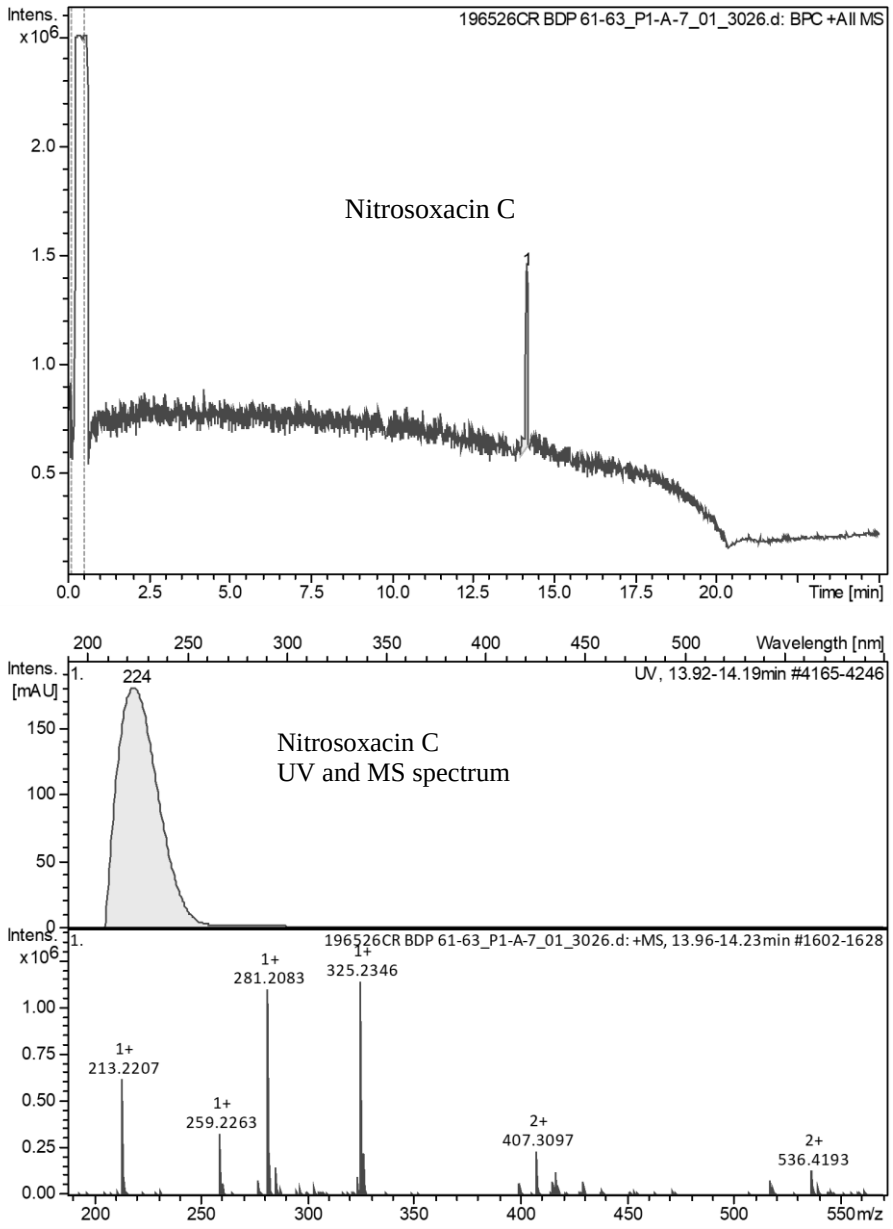


Figure 24. Chromatogram, UV and MS spectrum of Nitrosoxacin C found in the biomass extract of strain 196526CR.

3.6 Taxonomic study of *Streptomyces* sp. ASO4wet

3.6.1 Morphology and physiology

After incubation for four weeks, strain ASO4wet formed aerial mycelium with no spore was detected on ISP3 agar (Figure 25). From the physiological study, it was found that strain ASO4wet^T grew at 15-37^{o}C (optimum at 25-30^{o}C) and pH 6-9 (optimum at pH 7). Antibiotic susceptibility test suggested that isolate ASO4wet was sensitive to ampicillin (10 µg/disc), erythromycin (15 µg/disc), gentamycin 30 (µg/disc), penicillin G (6 µg/disc), tetracycline (30 µg/disc), vancomycin (30 µg/disc), and rifampicin (5 µg/disc); nevertheless, it was resistant to cefotaxime (30 µg/disc).

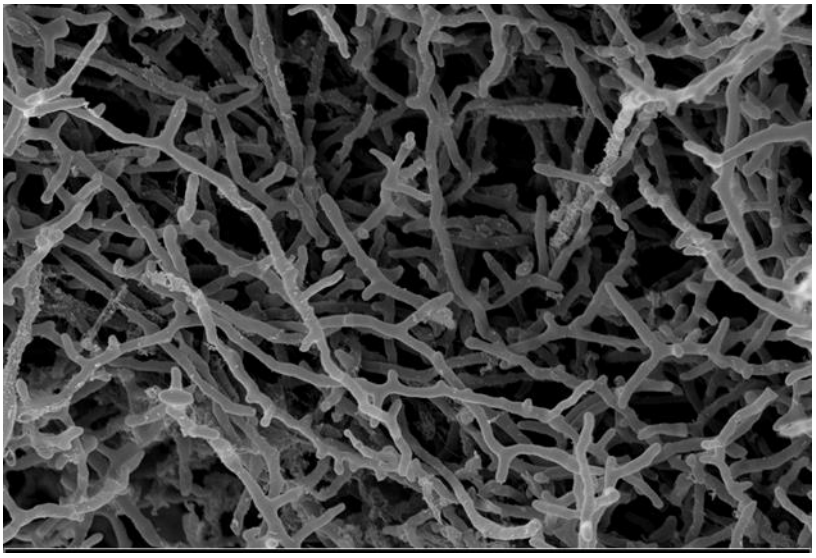


Figure 25. Scanning electron micrographs of aerial mycelium with no spore detected of strain ASO4wet^T after incubation on ISP 3 agar for 4 weeks at 30°C.

3.6.2 Chemotaxonomy

The cell-wall of isolate ASO4wet^T contained LL-diaminopimelic acid. Glucose and xylose were detected in its whole-cell hydrolysates (Figure 26). The identified fatty acids in strain ASO4wet^T were iso-C16:0 (35.01%), anteiso-C15:0 (21.97%), iso-C15:0 (13.75%), anteiso-C17:0 (8.80%), and iso-C14:0 (6.31%) (Figure 27). Menaquinone MK-9(H8) and MK-9(H6) were detected in a ratio of 12:1 (Figure 28). The polar lipid compositions were identified as diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidyl-N-methyl-ethanolamine, phosphatidylinositol mannoside, and four unidentified polar lipids (Figure 29).

3.6.3 16S rRNA gene analysis

By using BLAST or basic local alignment search tool from NCBI server (http:// www.ncbi.nlm.nih.gov/BLAST/)[179], it was founded that isolate ASO4wet^T belongs to the genus *Streptomyces*. The strain was closely related to *Streptomyces karpasiensis* K413^T (98.87%), *Streptomyces glycovorans* YIM M 10366^T (98.38%), and *Streptomyces abyssalis* M 10400^T (97.53%). It was located in the clade together with *Streptomyces karpasiensis* K413^T and *Streptomyces glycovorans* YIM M 10366^T that was supported by 89% bootstrap value in the neighbour-joining tree based on the 16S rRNA gene sequence (Figure 30). The relationship between these strains in the phylogenetic tree was also retrieved by using maximum-likelihood and maximum-parsimony algorithm.

3.6.4 Full genome analysis

The draft genome of strain ASO4wet^T was derived from PacBio-Sequencing. There was in total 7,377,472 bp was measured and the genome coverage was 117x. The genome was assessed by using Illumina MiSeq data and it encodes for about 6,500 genes after Prokka annotation. The G+C content was found to be 70.24 mol%.

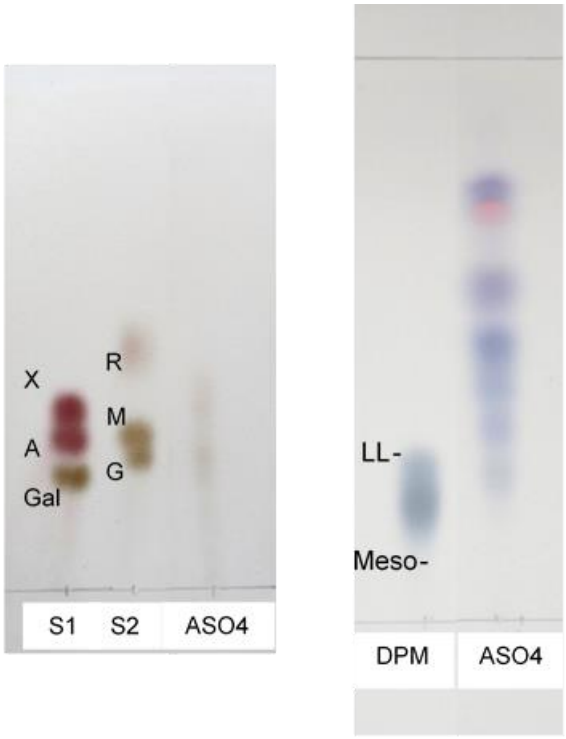
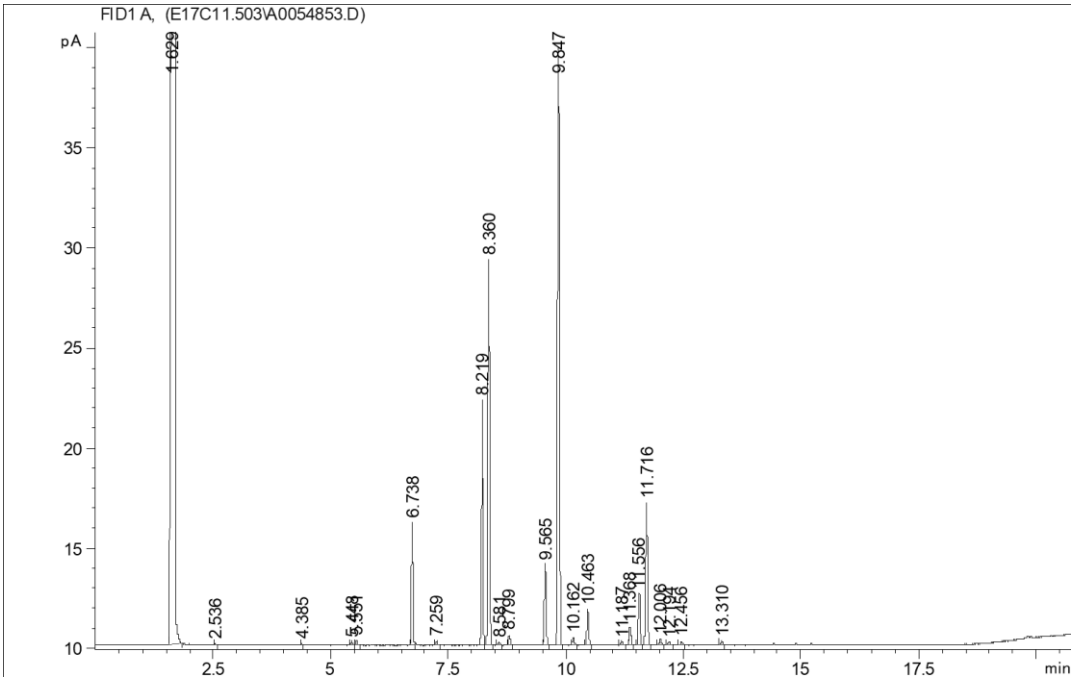
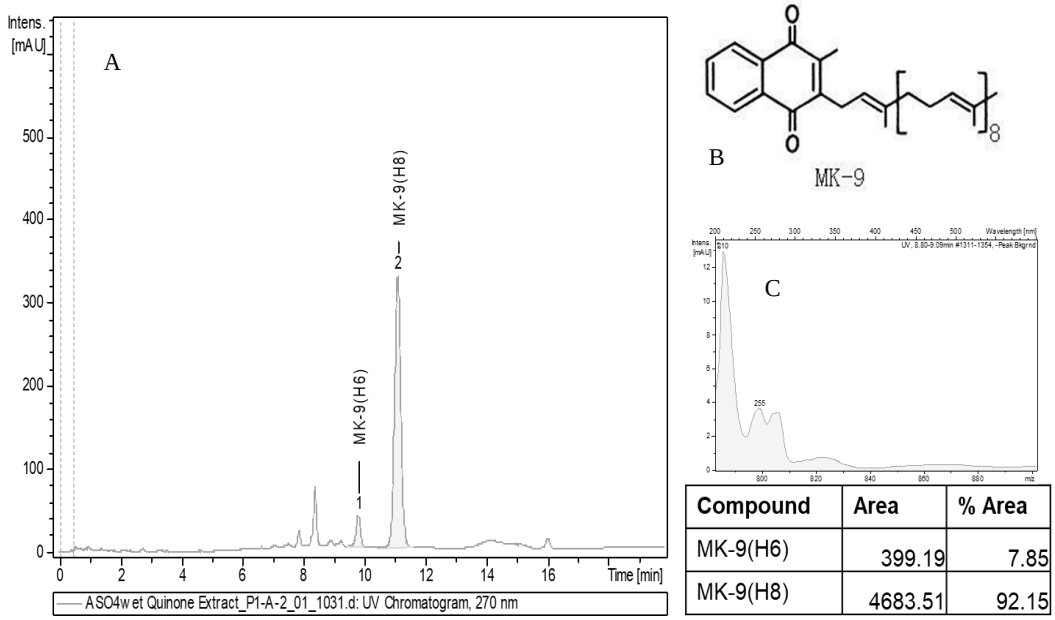


Figure 26. TLC chromatogram of whole-cell sugar analysis (left) and analysis amino acid of the cell wall (right) of isolate ASO4wet. S1: Standard 1; S2: Standard 2; X: Xylose; A: Arabinose; Gal: Galactose; DPM: Diaminopimelic acid standard; LL-: LL-Diaminopimelic acid; Meso: meso-Diaminopimelic acid; ASO4: isolate ASO4wet.



RT	Response	Ar/H	RFact	ECL	Peak Name	Percent
1.629	3.696E+8	0.026	----	7.010	SOLVENT PEAK	----
2.536	137	0.023	----	8.810		----
4.385	334	0.027	1.064	11.608	12:0 ISO	0.08
5.448	830	0.036	1.020	12.612	13:0 ISO	0.20
5.551	906	0.033	1.017	12.701	13:0 ANTEISO	0.22
6.738	**26965**	**0.035**	**0.986**	**13.617**	**14:0 ISO**	**6.31**
7.259	863	0.037	0.976	13.998	14:0	0.20
8.219	**60288**	**0.038**	**0.962**	**14.622**	**15:0 ISO**	**13.75**
8.360	**96480**	**0.039**	**0.960**	**14.714**	**15:0 ANTEISO**	**21.97**
8.581	628	0.035	0.958	14.857	15:1 B	0.14
8.799	2495	0.040	0.955	14.999	15:0	0.56
9.565	21520	0.042	0.948	15.459	16:1 ISO H	4.84
9.847	**156126**	**0.042**	**0.946**	**15.628**	**16:0 ISO**	**35.01**
10.162	1862	0.040	0.943	15.817	16:1 CIS 9	0.42
10.463	9394	0.043	0.941	15.998	16:0	2.10
11.187	933	0.042	0.937	16.417	16:0 9? METHYL	0.21
11.368	4876	0.044	0.937	16.521	17:1 ANTEISO C	1.08
11.556	14143	0.043	0.936	16.629	17:0 ISO	3.14
11.716	**39661**	**0.045**	**0.935**	**16.722**	**17:0 ANTEISO**	**8.80**
12.006	1619	0.047	0.934	16.889	17:0 CYCLO	0.36
12.194	847	0.044	0.934	16.998	17:0	0.19
12.456	956	0.046	0.933	17.147	16:0 ISO 3OH	0.21
13.310	1004	0.041	0.932	17.632	18:0 ISO	0.22

Figure 27. GC chromatogram of fatty acid analysis of isolate ASO4wet.



Compound	Area	% Area
MK-9(H6)	399.19	7.85
MK-9(H8)	4683.51	92.15

Figure 28. Menaquinones detected in isolate ASO4wet. A: Chromatogram from LC-MS of detected menaquinones; B: structure of menaquinone-9 (MK-9); C: UV Spectrum of menaquinone.

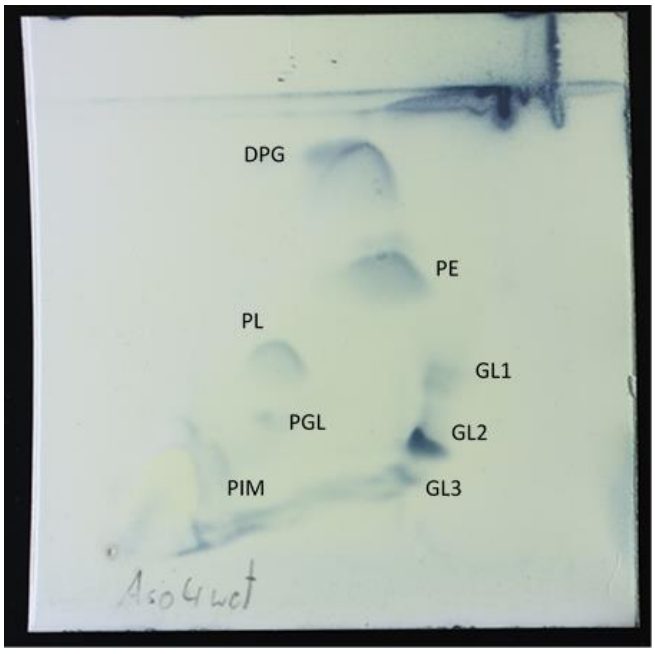


Figure 29. Polar lipid observed in strain ASO4wet. DPG: diphosphatidylglycerol; PE: phosphatidethanolamine; PL: unknown phospholipid; GL1-3: unknown glycolipid; PGL: unknown phosphoglycolipid; PIM: phosphatidylinositol mannoside.

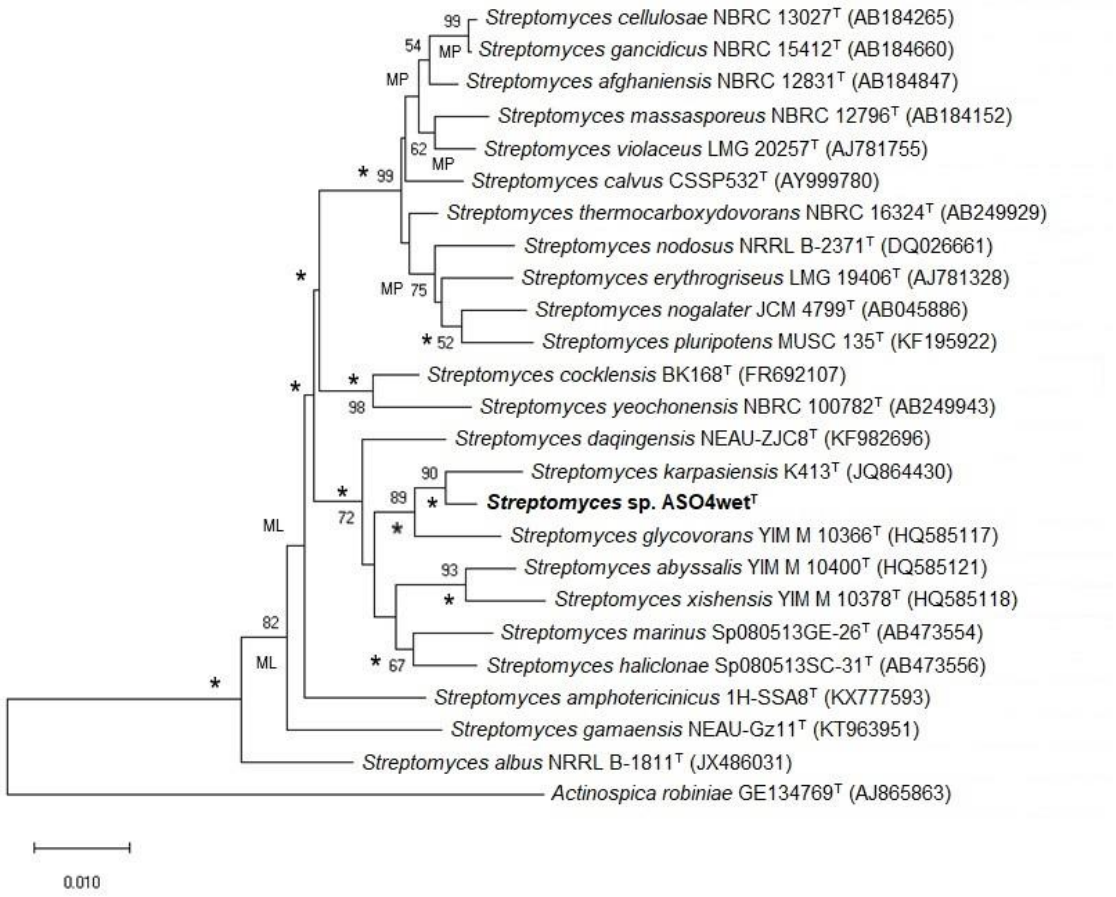


Figure 30. Neighbour-joining tree based on 16S rRNA gene sequences (1408 positions in the final dataset) showing relationships between *Streptomyces* sp. ASO4wet^T and its closely related *Streptomyces* species. The evolutionary distances were determined by using the Kimura 2-parameter method[175]. Asterisks mean branches of the tree that were also found using the maximum-likelihood and maximum-parsimony tree-making algorithms. ML and MP specify nodes that were also recovered using the maximum-likelihood and maximum-parsimony respectively. Numbers at the nodes are percentage bootstrap values based on a neighbour-joining analysis of 1,000 replicates, only values above 50 % are shown. Bar 0.010 substitutions per nucleotide position.

4 Discussion

4.1 Isolated Actinobacteria and their bioactivity

In the isolation of Actinobacteria, the soil samples were treated by heating at 60°C for 30 minutes before they were applied to the selection medium. Many vegetative bacterial cells are killed at 60°C. It is known that many vegetative yeast cells are more heat-sensitive than the vegetative bacterial cells and it is also previously reported that typically Gram-positive bacteria are more heat resistant than Gram-negative bacteria[180]. Some Actinobacteria living in soil, such as *Actinomyces*, *Streptomyces*, and *Micromonospora* form spores that can resist heat stress. *Streptomyces*, for example, are able to produce numerous hydrophobic spores that are moderately dense coat, contain small protective molecules like some group of sugars (trehalose), and consist of heat shock proteins[181].

The selection medium (5336 medium) for Actinobacteria isolation contains starch and casein. The combination of starch as the carbon source and casein as the nitrogen source was reported previously as one of the best media that permits good growth for *Streptomyces*. Moreover, it is also known that most of *Streptomyces* strains can use starch as the carbon source[182]. The medium also comprises magnesium sulfate ($MgSO_4$), which is essential for the cell division of the bacteria. Magnesium ion is more needed for Gram-positive bacteria than for Gram-negative bacteria. This probably because Gram-positive bacteria incorporate the magnesium ion into the complex structure of their cell wall[183].

Nalidixic acid, an antibacterial compound, is also one of the components in the selection medium. It inhibits DNA replication in some bacteria, such as *Escherichia coli* and *Bacillus substilis*[184]. It is known as a selective, immediate, and reversible inhibitor of bacterial DNA synthesis[185]. The other antimicrobial compound used in the selection medium is cycloheximide. Cycloheximide is a glutarimide derivative that has property as an antifungal agent by inhibiting protein synthesis. The compound binds the ribosome and blocks the elongation phase of translation process[186,187]. Some studies have previously reported the usage of these antimicrobial agents in the selection media for some Actinobacteria isolation[188,189].

In this study, plenty of Actinobacteria were isolated by using the preheated method and 5336 medium. The contamination from fungi and other bacteria could also be reduced with the addition of antifungal and antibacterial agents. Many of the isolated

Actinobacteria in this investigation belongs to *Streptomyces*. About 70% of the characterized strains were identified as *Streptomyces* and from the identified *Streptomyces*, only 27% of them had less than 99.50% similarity from the type strains based on 16S rRNA gene analysis. One of those is isolate SHP 1-2 that had 99.03% similarity to *Streptomyces viridochromogenes* NBRC 3113T. The extract produced by strain SHP 1-2 from 5294 medium could inhibit moderately the growth of *Staphylococcus aureus* and strongly inhibit the growth of *Bacillus substilis* and *Micrococcus luteus*.

For other strains that were identified closely to non-*Streptomyces* type strains according to the 16S rRNA analysis, there are 24% of them, which had less than 99.5% similarity from the type strain. One of the isolated non-*Streptomyces* strain is isolate 196526CR, which had 98.85% similarity to *Amycolatopsis thermalba* SF45T (98.85%). It produced an extract from 5294 medium that could inhibit, although moderately, the growth of *Bacillus subtilis, Staphylococcus aureus, Mucor hiemalis,* and *Candida albicans*. From the isolated strains that can yield extracts with moderate and strong activity against some tested microbes, almost 70% of them are *Streptomyces* strains.

Ten isolated strains showed strong activity against hepatitis C virus (HCV). Three of them were closely related to *Kitasatopora* species according to the result from EzBioCloud server (https://www.ezbiocloud.net)[156]. These strains are isolate DHE 2-1, MAE 1-11, and C190221. *Kitasatospora* and *Streptomyces* are very closely related because both of them belong to the same family Streptomycetaceae. They also have a similar lifestyle and morphology. In the capability of producing bioactive compounds, *Kitasatopora* could also be equivalent to *Streptomyces*. The difference between these two genera is on their cell-wall peptidoglycan composition. The cell-wall peptidoglycan of *Streptomyces* comprises LL-diaminopimelic acid (DAP), whereas *Kitasatospora* contains not only LL-diaminopimelic acid (LL-DAP) but also meso-DAP[190].

4.2 Description of strain SHP 1-2 and its secondary metabolites

Strain SHP 1-2 was isolated from soil collected in Enggano Island, Indonesia. The isolate has a broadly branched substrate mycelium that bears aerial hyphae which develop into spiral chains of smooth-surfaced spores on ISP 3 agar medium. It is a mesophilic, halotolerant, and non-melanin producing bacterium. The 16S rRNA gene

analysis suggested that isolate SHP 1-2 is close related to *Streptomyces* species. It has high G+C content in its genomic DNA. The morphologic and chemotaxonomic characteristics of strain SHP 1-2 have corresponding profiles to some other *Streptomyces* species that have been previously reported [191–193]. Therefore, it is determined that strain SHP 1-2 belongs to *Streptomyces* species.

In the phenotypic characteristic, many differences are found between strain SHP 1-2 and the closest relative strains based on 16S rRNA gene sequence, i.e., *Streptomyces malachitofuscus* DSM 40332^T (99.03%), *Streptomyces viridochromogenes* DSM 40110^T (99.03%), and *Streptomyces misionensis* DSM 40306^T (98.96%), as well as the closest one from the MLSA distance (*Streptomyces fumigatiscleroticus* DSM 43154^T, MLSA distance: 0.058). The significant difference is in the basis of single carbon source usage, as for strain SHP 1-2, it can only use glucose, while the others are able to use several of sole carbon sources. Furthermore, based on the enzymatic system, strain SHP 1-2 shows a substantial distinction with the others. The chymotrypsin activity was not detected in strain SHP 1-2, while in the others, the chymotrypsin activities are observed. It can be inferred; therefore, that strain SHP 1-2, in the phenotypic features, is different from the compared type strains.

Recently, however, there are some problems to delineate *Streptomyces* strains. This because of the vast amounts of isolates and insufficiently determined species, the weak resolution of 16S rRNA gene sequence as the phylogenetic standard, complexities in comparing essential phenotypic features between the strains, and inconvenience of DNA–DNA hybridization (DDH) and DNA fingerprinting for rapid analysis. Multilocus sequence analysis (MLSA) has been used recently in microbial systematic. MLSA has been recently used for delineation of *Streptomyces* species because of its robustness in the molecular method[130].

By using five house-keeping genes, i.e., *atpD, gyrB, recA, rpoB* and *trpB* in MLSA analysis, it was found that strain SHP 1-2 showed to have MLSA distance more than 0.007 with all of the other related species. The MLSA distance of 0.007 is equivalent to 70 % DNA–DNA homology[130]. The MLSA distance that is more than 0.007 means that the strain can be separated with the other compared strains. After studying polyphasic taxonomy, that includes the genotypic and phenotypic comparison between strain SHP 1-2 and the close strains, it can be concluded that the strain SHP 1-2 is a novel species in the *Streptomyces* genus.

Piericidin A1 is the most active compound produced by strain SHP 1-2. This compound is also produced by other *Streptomyces* species such as *Streptomyces mobaraensis* and *Streptomyces pactum*[194]. The mechanism of the activity against some tested organisms is suggested due to its ability to inhibit mitochondrial and bacterial NADH-ubiquinone oxidoreductases[195]. It was also previously reported that the compound possesses the antibacterial activity against Gram-negative bacteria *Chromobacterium violaceum* CV026 by inhibiting the quorum-sensing mechanism[196].

Two new molecules that were isolated from strain SHP 1-2 are indolactam derivatives. These compounds have a moderate activity to inhibit the proliferation of KB-3-1 and PC-3 cells lines. As for indolactam variant II, it also has modest activity against SK-OV-3 cell line. The other indolactam structure, indolactam V, was also isolated from strain SHP 1-2. It has been reported previously that indolactam V has numerous biological activities. It can stimulate protein kinase C (PKC), generate pancreatic progenitor cells from human embryonic stem cells (hESCs), and induce the Epstein-Barr virus early antigen (EBV-EA) of Raji cells (B lymphocyte)[197–199]. This compound was previously also isolated from *Streptomyces blastmyceticum* NA3417 and known as a biosynthetic intermediate of teleocidins, which are identified as influential skin tumor promoters[200].

Two other compounds produced by strain SHP 1-2 are cyclo(phenylalanyl-prolyl) and lyngbyatoxin A. Cyclo(phenylalanyl-prolyl) is diketopiperazine molecule that was also isolated previously from *Streptomyces* sp. (NPS008187)[201]. The other study suggested that cyclo(phenylalanyl-prolyl) could be produced by *Lactobacillus plantarum* strain (MiLAB 393). The compound was also reported having biological activity property as antifungal agent[202]. As for lyngbyatoxin A, this compound was first reported in the previous study to be found in marine cyanobacterium *Moorea producens* (formerly *Lyngbya majuscula*). It is the causative compound of seaweed dermatitis. It has a highly inflammatory effect, vigorous tumor-promoting activity and capability to stimulate protein kinase C isozymes. The compound was determined to be the same as teleocidin A-1, which was isolated from *Streptomyces mediocidicus*[203].

4.3 Description of strain MAE 1-11 and its secondary metabolites

The calculation from EzBioCloud server (https://www.ezbiocloud.net)[156] using 16S rRNA gene suggested that strain MAE-11 was very close to the type

strain *Kitasatospora albolonga* NBRC 13465^T (99.93%). The phylogenetic tree analysis result, however, indicated that strain MAE-11 was closer to *Streptomyces cavourensis* NBRC 13026^T than to *K. albolonga* NBRC 13465^T according to the neighbour-joining algorithm. The clade was also supported by a relatively high percentage of bootstrap value.

Kitasatospora is the genus of Actinobacteria, which was firstly proposed in 1982 and the name itself refers to Kitasato, who was a Japanese bacteriologist[98]. *Kitasatospora* and *Streptomyces* are very closely related in the taxonomy as both of them are in the same family Streptomycetaceae and also look similar in their morphology. The thing that makes them different is the cell-wall composition. *Streptomyces* contains LL-diaminopimelic acid and no meso-diaminopimelic acid, whereas *Kitasatopora* has both of the LL- and meso-diaminopimelic acid[204]. The other thing which different is that *Kitasatopora* has galactose as the diagnostic sugar, while *Streptomyces* has no diagnostic sugar[125].

Strain MAE 1-11 was isolated from soil collected in the mangrove area of Enggano Island, Indonesia. It is a halotolerant and melanin producer bacterium. Strain MAE 1-11 can be distinguished from *K. albolonga* NRRL B-3604^T (= NBRC 13465^T) on the basis of the enzymatic system. *K. albolonga* NRRL B-3604^T (= NBRC 13465^T) does not have activity for phosphatase alkaline, esterase (C4), alpha-chymotrypsin, and alpha-glucosidase, while strain MAE 1-11 have all these enzymes[205,206]. Strain MAE 1-11 is also different from *S. cavourensis* NBRC 13026^T according to the enzymatic activity. Some enzymes were not detected in *S. cavourensis* NBRC 13026^T, such as esterase (C8), trypsin, phosphatase acid, naphtol-AS-BI-phosphohydrolase, and beta-galactosidase[206], whereas isolate MAE 1-11 equipped with these enzymes.

The primary compound produced by strain MAE 1-11 is bafilomycin D. The compound has antiviral activity against HCV. Bafilomycin D is a 16-membered ring macrolide antibiotic that possesses activity in the inhibition of V- and P-ATPases, although it is not as strong as bafilomycin A1. V-ATPases are vacuolar-type, proton-translocating ATPases (V-ATPases), while P-ATPases are ATPases with phosphorylated states (P-ATPases). Both of them can be found in animal and plant cells, as well as in yeast, fungi, and bacteria[207]. Bafilomycin D was also previously isolated from *Streptomyces griseus* Tü 2599, *Streptomyces sp.* YIM56209,

Streptomyces albolongus (novel name is *Kitasatospora albolonga*) strain YIM 101047[208–210].

The mechanism of antiviral bafilomycin D against HCV may be similar to how bafilomycin A1 can inhibit influenza A virus (IAV) replication. Bafilomycin A1 at relatively high concentration (≥10 nM) was reported that it could inhibit V-ATPase and decrease endosome acidification as well as lysosome number; consequently, it diminished IAV replication. However, it could also trigger cytotoxicity for the host cell. Interestingly, at a shallow concentration (0.1 nM), this molecule was still able to inhibit the replication, and the release of IAV albeit this amount of bafilomycin A1 is neither causing reduction of lysosome number nor toxic to the host cell[211].

4.4 Description of strain 196526CR and its secondary metabolites

The 16S rRNA gene analysis, according to the result from EzBioCloud server (https://www.ezbiocloud.net)[156], suggested that strain 196526CR was very close to the type strain *Amycolatopsis thermalba* SF45^T (98.85%), however, based on the constructed phylogenetic tree, *Amycolatopsis deserti* GY024^T was the closest strain to strain 196526CR. The clade formed in the phylogenetic tree between strain 196526CR and *A. deserti* GY024^T was found both with the neighbour-joining and maximum-likelihood method, although in neighbor-joining method, this clade was only supported by less than 50% of bootstrap values.

Amycolatopsis is one of the genera in Actinobacteria and belongs to the family Pseudonocardiaceae[212]. Until now, the genus *Amycolatopsis* includes already 76 published species (http://www.bacterio.net/amycolatopsis.html). *Amycolatopsis* can be distinguished from the other genera in the family Pseudonocardiaceae by using genotypic and phenotypic assessment. It has no sporangia; no motile spores; the diagnostic sugars are arabinose and galactose; the phospholipid system contains PE (phosphatidylethanolamine), DPG (diphosphatidylglycerol), PG (phosphatidyl-glycerol), and PI (phosphatidylinositol; and the predominant menaquinone is MK-9(H4)[213]. The spores in *Amycolatopis* can be found both in aerial and substrate hyphae. The spores in aerial hyphae are formed in chains, the genus also has fragmented substrate hyphae[214] and contains meso-diaminopimelic acid in its cell-wall structure[212]. The genus-specific primers for *Amycolatopsis* were also constructed in the previous study for detecting *Amycolatopsis* in soil samples[215].

Some antibiotics are produced by genus *Amycolatopsis*. Species *Amycolatopsis orientalis* is the producer of vancomycin, which is a strong glycopeptide antibiotic that has activity against methicillin-resistant *Staphylococcus aureus* (MRSA) infections[216]. Balhimycin, which is categorized to the vancomycin class of glycopeptide antibiotic, is produced by *Amycolatopsis Amycolatopsis balhimycina*. Balhimycin was found to be correspondingly strong as vancomycin against MRSA strains and more powerful than vancomycin, specifically against *Clostridium* strains[217]. *Amycolatopsis mediterranei* is well-known as a producer of Rifamycin B, which is used for the treatment of tuberculosis and other diseases caused by *Mycobacterium*. Rifamycin B belongs to the ansamycin family, which is characterized by a macrocyclic structure containing aromatic moiety bridged by an aliphatic ansa chain[218,219].

Strain 196526CR was isolated from soil in Bali, Indonesia. The strain is a halotolerant and non-melanin producing bacterium. The isolate can grow well by using glucose and fructose as a single carbon source, however, there was no alpha-glucosidase activity detected in this strain. Alpha-glucosidase is one of the essential enzymes in starch hydrolysis to produce glucose[220]. This may be the reason why the strain can not grow in ISP 4 medium, which contains only starch as the single source of carbon.

Not like strain 196526CR, one of the close strains, *A. deserti* GY024^T, have no enzymatic activity in beta-glucosidase. Another close type strain, *A. thermalba* SF45^T were found not having N-acetyl-beta-glucoseamidase and acid phosphatase[221], whereas, for the strain 196526CR, these enzymes are produced. This finding may suggest that strain 196526CR can probably be distinguished from *A. deserti* GY024^T and *A. thermalba* SF45^T based on the enzymatic system.

Some compounds that are produced by strain 196526CR are coproporphyrin III, zinc coproporphyrin III, and nitrosoxacin C. Coproporphyrin III is a tetrapyrrole moiety and is the primary intermediate of heme biosynthesis in *Amycolatopsis* and other Actinobacteria. Heme is very important in the numerous biological process. It functions as a prosthetic group for various proteins, such as cytochromes, globins, catalases, peroxidases, and transporters. These proteins are essential in respiration, photosynthesis, metabolism, and transport of oxygen in organisms[222,223].

Zinc coproporphyrin III is the derivative of coproporphyrin III which is bound to zinc metal. The previous study suggested that coproporphyrin III showed a higher affinity

for zinc than other metals[224]. The compound was also found in *Streptomyces* sp. AC8007 and has some biological activities, such as a histamine-release inhibitor and a potent photosensitizer for controlling tumor growth in photodynamic cancer therapy[225].

Nitrosoxacin C was isolated by using bioassay-guided isolation against *Staphylococcus aureus* since the molecule was difficult to be detected by the UV detector. The MIC results suggest that Nitrosoxacin C has moderate antimicrobial activity against *Staphylococcus aureus* and *Bacillus substilis*. It is not known previously that Nitrosoxacin C has antibacterial activity against these bacteria. The previous study reported that Nitrosoxacin C was isolated from *Streptomyces* sp. AA4091 and it is known to have the activity as a 5-lipoxygenase inhibitor. The possible mechanism of 5-lipoxygenase inhibitory activity is because of its chelating activity[226]. 5-lipoxygenase inhibitors are used in the treatment of inflammatory bowel disease (IBD)[227].

4.5 Description of strain ASO4wet

Strain ASO4wet was isolated from the sponge in a deep-sea collected from the North Atlantic Ocean. Some of the characterizations of this strain were conducted previously by Landwehr[147]. These include 16S rDNA analysis, morphology and melanin production, some physiological studies, MALDI-TOF analysis by using ribosomal protein, DNA-DNA hybridization, and ribotyping analysis with some closely related strains. This recent study reports the other characteristics such as morphology by scanning electron microscopy (SEM), some physiology studies, chemotaxonomy assessment, phylogenetic tree construction based on 16S rRNA gene, and full genome sequencing for determining the size of the genome as well as the G+C content.

Strain ASO4wet is an aerobic and Gram-positive bacterium that forms branched substrate mycelium with the aerial hyphae that can be seen in ISP3 and ISP7 agar medium. The strain grows well on ISP2, ISP3, ISP4, and ISP5 after two weeks incubation at 30°C. The enzymatic system comprises esterase (C4), trypsin, chymotrypsin, β-galactosidase, and α-glucosidase. The NaCl tolerance of this strain is 0-10% (w/v) NaCl. The strain can grow with glucose, arabinose, sucrose, xylose, inositol, mannitol, fructose, rhamnose, and raffinose as the sole carbon sources[147].

Spores were not observed in ISP3 even after four weeks incubation at 30°C. The optimum growth was detected at 25–30°C and at pH 7. From the 16S rRNA and chemotaxonomy analysis results as well as the high G+C content in its genomic DNA, it is determined that strain ASO4wet is a member of the genus *Streptomyces*. Strain ASO4wet^T formed a stable subclade with *Streptomyces karpasiensis* K413^T with high bootstrap value and adjacent to *Streptomyces glycovorans* YIM M 10366^T. The position in the phylogenetic tree between these *Streptomyces* strains was also confirmed by the maximum-likelihood and maximum-parsimony methods.

Strain ASO4wet can be separated from its closest strains based on the enzymatic system such as lipase (C14), beta-galactosidase, beta-glucosidase, the use of single carbon, MALDI-TOF, and ribotyping analysis. Furthernore, the DNA-DNA hybridization results with the closest *Streptomyces* type strains are lower than 70%[147]. Therefore, it is suggested that strain ASO4wet represents a novel species within the genus *Streptomyces*.

5 Summary

Hundreds of Actinobacteria were successfully isolated from Indonesian source samples. However, due to the limitation of time allocation, only around half of them were characterized. The study was focused on strain SHP 1-2, MAE 1-11, and 19626CR due to its ability to produce active extracts against either some microbes or hepatitis C virus (HCV). Another strain that was used in this study was strain ASO4wet that was isolated previously from deep-sea in the North Atlantic Ocean. Strain SHP 1-2 was known to produce two novel indolactam derivatives. It was also suggested that strain SHP 1-2 is the novel species in the genus *Streptomyces*. Bafilomycin D, which was produced by strain MAE -11, was revealed to have antiviral properties against HCV. This strain is closely related to *Kitasatopora* and *Streptomyces* species. Strain 196526CR, which was characterized as *Amycolatopsis* species, generated nitrosoxacin C that has antimicrobial property. The antimicrobial activity of nitrosoxacin C was firstly reported in this study. A chemotaxonomic study of strain ASO4wet gave supporting data that this strain belongs to the genus *Streptomyces*.

Streptomyces species can typically be detected by their aerial mycelium formation. However, some non-*Streptomyces* genera such as *Kitasatospora*, *Amycolatopsis*, and *Pseudonocardiopsis* also have similar aerial mycelium as *Streptomyces*. The chemotaxonomic study, especially for amino acid of cell-wall analysis, can distinguish between *Streptomyces* and non-*Streptomyces* species based on the present of LL-diaminopimelic acid and its isomer. The identification based on 16S rRNA sequence further is needed to clarify which type strain the isolates are close to. Around half of the isolates were not studied yet either for taxonomic characterization or for metabolite production. Further study is needed for clarifying the species and its secondary metabolites.

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7 Appendix

Table S 1. List of Actinobacteria strains isolated from Indonesian samples

No.	Strain	Samples location	No.	Strain	Samples location
1	4421	Beach, Bali	99	196831CR	Botanical Garden, Bali
2	4422	Beach, Bali	100	196833CR	Botanical Garden, Bali
3	4423	Beach, Bali	101	196921CR	Botanical Garden, Bali
4	4431	Beach, Bali	102	196932CR	Botanical Garden, Bali
5	4433	Beach, Bali	103	197011CR	Botanical Garden, Bali
6	4435	Beach, Bali	104	197012CR	Botanical Garden, Bali
7	5931	Lava beach, Bali	105	197013CR	Botanical Garden, Bali
8	19821	Botanical Garden, Bogor, West Java	106	197014CR	Botanical Garden, Bali
9	19823	Botanical Garden, Bogor, West Java	107	197017CR	Botanical Garden, Bali
10	19824	Botanical Garden, Bogor, West Java	108	197019CR	Botanical Garden, Bali
11	190221	Forest low altitude, Kendari, Southeast Sulawesi	109	197212CR	Botanical Garden, Bali
12	190224	Forest low altitude, Kendari, Southeast Sulawesi	110	1982-1	Botanical Garden, Bogor, West Java
13	190225	Forest low altitude, Kendari, Southeast Sulawesi	111	1982-3	Botanical Garden, Bogor, West Java
14	190231	Forest low altitude, Kendari, Southeast Sulawesi	112	1982-3 (orange)	Botanical Garden, Bogor, West Java
15	190232	Forest low altitude, Kendari, Southeast Sulawesi	113	1982-4	Botanical Garden, Bogor, West Java
16	190233	Forest low altitude, Kendari, Southeast Sulawesi	114	198311CR	Botanical Garden, Bogor, West Java
17	190234	Forest low altitude, Kendari, Southeast Sulawesi	115	198331CR	Botanical Garden, Bogor, West Java
18	190235	Forest low altitude, Kendari, Southeast Sulawesi	116	198332CR	Botanical Garden, Bogor, West Java
19	190401	Mangrove, Kendari, Southeast Sulawesi	117	198333CR	Botanical Garden, Bogor, West Java
20	194601	Cultural Park, Bali	118	198334CR	Botanical Garden, Bogor, West Java
21	195105	Cultural Park, Bali	119	198335CR	Botanical Garden, Bogor, West Java
22	195107	Cultural Park, Bali	120	198414CR	Botanical Garden, Bogor, West Java
23	1951016	Cultural Park, Bali	121	1-SO1	Mangrove, Jakarta
24	180811CR	Malang, East Java	122	2112-2	Mangrove, Jakarta
25	180812CR	Malang, East Java	123	2112-SO	Mangrove, Jakarta

26	180813CR	Malang, East Java	124	2113SO	Mangrove, Jakarta
27	180814CR	Malang, East Java	125	2114-1SO	Mangrove, Jakarta
28	1808210CR	Malang, East Java	126	2115SO	Mangrove, Jakarta
29	180821CR	Malang, East Java	127	2118-1	Mangrove, Jakarta
30	180822CR	Malang, East Java	128	2118-3	Mangrove, Jakarta
31	180823CR	Malang, East Java	129	2118SO	Mangrove, Jakarta
32	180824CR	Malang, East Java	130	2120SO	Mangrove, Jakarta
33	180825CR	Malang, East Java	131	2122-2	Mangrove, Jakarta
34	180826CR	Malang, East Java	132	2122-5	Mangrove, Jakarta
35	180827CR	Malang, East Java	133	2123-1BSO	Mangrove, Jakarta
36	180828CR	Malang, East Java	134	2124-1SO	Mangrove, Jakarta
37	180829CR	Malang, East Java	135	2125-2 (grey)	Mangrove, Jakarta
38	189921CR	Forest low altitude, Kendari, Southeast Sulawesi	136	2125-2 (white)	Mangrove, Jakarta
39	189923ACR	Forest low altitude, Kendari, Southeast Sulawesi	137	2125-3	Mangrove, Jakarta
40	189923BCR	Forest low altitude, Kendari, Southeast Sulawesi	138	2125-5	Mangrove, Jakarta
41	190122BCR	Forest low altitude, Kendari, Southeast Sulawesi	139	2126-1SO	Mangrove, Jakarta
42	190131ACR	Forest low altitude, Kendari, Southeast Sulawesi	140	2126-2SO	Mangrove, Jakarta
43	190222CR	Forest low altitude, Kendari, Southeast Sulawesi	141	2126-3SO	Mangrove, Jakarta
44	190224CR	Forest low altitude, Kendari, Southeast Sulawesi	142	2126SO	Mangrove, Jakarta
45	190227CR	Forest low altitude, Kendari, Southeast Sulawesi	143	2126SO(3)	Mangrove, Jakarta
46	190711GyCR	Cimahi, West Java	144	2126SO-1	Mangrove, Jakarta
47	190712WCR	Cimahi, West Java	145	2126SO-2	Mangrove, Jakarta
48	190721CR	Cimahi, West Java	146	2127SO	Mangrove, Jakarta
49	194813CR	Cultural Park, Bali	147	2-1MKBSO	Mangrove, West Kalimantan
50	194821CR	Cultural Park, Bali	148	2-2MKBSO	Mangrove, West Kalimantan
51	194822CR	Cultural Park, Bali	149	3-BBSO	Beach, Bali
52	194823CR	Cultural Park, Bali	150	4-1BKBSO	Beach, West Kalimantan
53	194831CR	Cultural Park, Bali	151	4-2BKBSO	Beach, West Kalimantan
54	194912CR	Cultural Park, Bali	152	4-3BKBSO	Beach, West Kalimantan
55	194921CR	Cultural Park, Bali	153	4-4BKBSO	Beach, West Kalimantan
56	194931CR	Cultural Park, Bali	154	4-5BKBSO	Beach, West Kalimantan
57	194933RCR	Cultural Park, Bali	155	4-6BKBSO	Beach, West Kalimantan
58	194934CR	Cultural Park, Bali	156	5-1BLSO	Beach, Lampung

59	194935CR	Cultural Park, Bali	157	5-2BLSO	Beach, Lampung
60	194938CR	Cultural Park, Bali	158	5-3BLSO	Beach, Lampung
61	195003R	Cultural Park, Bali	159	6-2BPSO	Beach, Papua
62	195211CR	Cultural Park, Bali	160	6BPSO	Beach, Papua
63	195213BCR	Cultural Park, Bali	161	7BPSO	Beach, Papua
64	195213CR	Cultural Park, Bali	162	9BLSSO	Beach, Lampung
65	195215CR	Cultural Park, Bali	163	BLH 12-3	Bitung, North Sulawesi
66	195217CR	Cultural Park, Bali	164	C190221	Forest low altitude, Kendari, Southeast Sulawesi
67	195218CR	Cultural Park, Bali	165	C194911	Cultural Park, Bali
68	195219CR	Cultural Park, Bali	166	C194922A	Cultural Park, Bali
69	1952211CR	Cultural Park, Bali	167	C1950A	Cultural Park, Bali
70	1952212CR	Cultural Park, Bali	168	C195212	Cultural Park, Bali
71	1952213CR	Cultural Park, Bali	169	C195311	Cultural Park, Bali
72	195222CR	Cultural Park, Bali	170	C195321A	Cultural Park, Bali
73	195224CR	Cultural Park, Bali	171	C196921	Botanical Garden, Bali
74	195226CR	Cultural Park, Bali	172	CA1-BKBSO	Beach, West Kalimantan
75	195227CR	Cultural Park, Bali	173	CA2-SO12	Mangrove, Jakarta
76	195227GnCR	Cultural Park, Bali	174	DHE 2-1	Enggano Island, Bengkulu
77	195228CR	Cultural Park, Bali	175	DHE 9-4	Enggano Island, Bengkulu
78	195231CR	Cultural Park, Bali	176	GBSL-9	Lampung, Indonesia
79	195232CR	Cultural Park, Bali	177	GKRL-2	Lampung, Indonesia
80	195233CR	Cultural Park, Bali	178	GKRL-3	Lampung, Indonesia
81	195321BCR	Cultural Park, Bali	179	GKRL-4	Lampung, Indonesia
82	195331CR	Cultural Park, Bali	180	MAE 1-11	Enggano Island, Bengkulu
83	195332CR	Cultural Park, Bali	181	MAE 1-3	Enggano Island, Bengkulu
84	195333CR	Cultural Park, Bali	182	SHP 1-2	Enggano Island, Bengkulu
85	195334CR	Cultural Park, Bali	183	SHP 1-4	Enggano Island, Bengkulu
86	195335CR	Cultural Park, Bali	184	SHP 1-5	Enggano Island, Bengkulu
87	195336CR	Cultural Park, Bali	185	SHP 1-6	Enggano Island, Bengkulu
88	195337CR	Cultural Park, Bali	186	SHP 2-2	Enggano Island, Bengkulu
89	195338CR	Cultural Park, Bali	187	SHP 2-4	Enggano Island, Bengkulu
90	195339CR	Cultural Park, Bali	188	SHP 2-5	Enggano Island, Bengkulu
91	1965114CR	Botanical Garden, Bali	189	SHP 6-2	Enggano Island, Bengkulu
92	196511CR	Botanical Garden, Bali	190	SHP 6-3	Enggano Island, Bengkulu
93	196512CR	Botanical Garden, Bali	191	SHP 6-4	Enggano Island, Bengkulu

94	196522CR	Botanical Garden, Bali	192	SHP 6-5	Enggano Island, Bengkulu
95	196523CR	Botanical Garden, Bali	193	SHP 6-6	Enggano Island, Bengkulu
96	196524CR	Botanical Garden, Bali	194	SHP 7-1	Enggano Island, Bengkulu
97	196526CR	Botanical Garden, Bali	195	SHP 7-3	Enggano Island, Bengkulu
98	196532CR	Botanical Garden, Bali	196	SHP 7-5	Enggano Island, Bengkulu

Table S 2. List of isolated Actinobacteria with the closest species based on 16S rRNA gene analysis

No.	Name of Strain	Closest type strain by 16S rRNA gene analysis	Similarity (%)	Completeness of the sequence (%)
	***Streptomyces* strain**			
1	9BLSSO	*Streptomyces lanatus*	97.03	99.9
2	195105	*Streptomyces cyaneus*	98.77	50.7
3	195107	*Streptomyces cyaneus*	98.77	50.6
4	195227GnCR	*Streptomyces filipinensis*	98.81	58.0
5	DHE 9-4	*Streptomyces spongiae*	98.83	100
6	190122BCR	*Streptomyces roietensis*	98.89	58.2
7	190233	*Streptomyces glomeratus*	98.96	33.3
8	SHP 1-2	*Streptomyces viridochromogenes*	99.03	99.9
9	198414CR	*Streptomyces cinnabarigriseus*	99.14	64.5
10	GKRL-2	*Streptomyces jiujiangensis*	99.17	100
11	198333CR	*Streptomyces griseochromogenes*	99.37	55.1
12	C194911	*Streptomyces spiralis*	99.40	58.2
13	SHP 6-4	*Streptomyces xylanilyticus*	99.45	100
14	194601	*Streptomyces viridiviolaceus*	99.45	50.1
15	GBSL-9	*Streptomyces jiujiangensis*	99.48	66.3
16	1965114CR	*Streptomyces pharetrae*	99.49	54.5
17	195337CR	*Streptomyces cadmiisoli*	99.51	56.3
18	196932CR	Streptomyces canus	99.52	58.0
19	190234	*Streptomyces misionensis*	99.52	28.7
20	197019CR	*Streptomyces antibioticus*	99.55	61.7
21	190235	*Streptomyces rhizosphaericus*	99.61	53.6
22	C194922A	*Streptomyces prasinosporus*	99.63	36.9
23	C195311	*Streptomyces olivaceus*	99.71	47.5
24	190224CR	*Streptomyces lannensis*	99.72	48.7
25	195003R	*Streptomyces tuirus*	99.75	55.9
26	198332CR	*Streptomyces gilvifuscus*	99.75	55.1
27	198335CR	*Streptomyces panaciradicis*	99.82	38.5
28	1951016	*Streptomyces xiangtanensis*	99.86	50.6
29	190232	*Streptomyces jiujiangensis*	99.88	58.2

30	195213BCR	*Streptomyces indiaensis*	99.88	58.2
31	GKRL-3	*Streptomyces hyaluromycini*	99.88	56.0
32	GKRL-4	*Streptomyces hyaluromycini*	99.89	64.0
33	4433	*Streptomyces albogriseolus*	100	58.6
34	190221	*Streptomyces lannensis*	100	48.7
35	190224	*Streptomyces katrae*	100	29.0
36	190225	*Streptomyces lucensis*	100	30.3
37	190231	*Streptomyces lannensis*	100	29.3
38	180822CR	*Streptomyces griseorubiginosus*	100	61.1
39	194813CR	*Streptomyces levis*	100	58.2
40	194823CR	*Streptomyces levis*	100	46.6
41	196511CR	*Streptomyces griseorubiginosus*	100	55.1
42	196512CR	*Streptomyces antibioticus*	100	58.8
43	2122-5	*Streptomyces badius*	100	52.3
44	C195321A	*Streptomyces levis*	100	54.9
45	C196921	*Streptomyces violaceolatus*	100	61.7
46	MAE 1-3	*Streptomyces albogriseolus*	100	52.9
47	SHP 1-4	*Streptomyces hydrogenans*	100	61.8
48	SHP 1-5	*Streptomyces hydrogenans*	100	48.7
49	SHP 1-6	*Streptomyces hydrogenans*	100	59.4
50	SHP 2-2	*Streptomyces hydrogenans*	100	29.9
51	SHP 2-4	*Streptomyces hydrogenans*	100	61.8
52	SHP 2-5	*Streptomyces olivaceus*	100	45.1
53	SHP 6-2	*Streptomyces hydrogenans*	100	57.8
54	SHP 6-3	*Streptomyces althioticus*	100	42.5
55	SHP 6-5	*Streptomyces hydrogenans*	100	61.8
56	SHP 6-6	*Streptomyces hydrogenans*	100	53.5
57	SHP 7-1	*Streptomyces albidoflavus*	100	68
58	SHP 7-3	*Streptomyces olivaceus*	100	42.2
59	SHP 7-5	*Streptomyces hydrogenans*	100	48.7
	Non-*Streptomyces* strain			
1	195336CR	*Mycobacterium palauense*	98.47	100
2	194938CR	*Kibdelosporangium banguiense*	98.74	100
3	196526CR	*Amycolatopsis thermalba*	98.85	100
4	2118-1	*Nocardia rhizosphaerae*	99.13	40
5	195334CR	*Amycolatopsis magusensis*	99.37	100
6	190401	*Microbispora hainanensis*	99.38	44.7
7	195232CR	*Couchioplanes caeruleus* subsp. *Caeruleus*	99.62	55.2
8	4431	*Nocardiopsis lucentensis*	99.78	62.2
9	4422	*Nocardiopsis lucentensis*	99.83	39.4
10	180824CR	*Amycolatopsis japonica*	99.80	35.5
11	4421	*Nocardiopsis lucentensis*	99.86	49.3
12	4423	*Nocardiopsis lucentensis*	99.86	49.3

13	4435	*Nocardiopsis lucentensis*	99.87	54.0
14	1982-4	*Nocardia araoensis*	99.87	52.7
15	1982-1	*Nocardia araoensis*	99.87	52.5
16	180813CR	*Nonomuraea endophytica*	99.89	61.5
17	MAE 1-11	*Kitasatospora albolonga*	99.93	100
18	1808210CR	*Nonomuraea endophytica*	100	58.9
19	5931	*Pseudonocardia kongjuensis*	100	63.6
20	1982-3	*Micromonospora wenchangensis*	100	59.8
21	4-5BKBSO	*Pseudonocardia carboxydivorans*	100	62.2
22	BLH 12-3	*Micromonospora aurantiaca*	100	29.9
23	C190221	*Kitasatospora putterlickiae*	100	46.6
24	C1950A	*Saccharothrix xinjiangensis*	100	29.2
25	DHE 2-1	*Kitasatospora albolonga*	100	59.0

Table S 3. List of strains that can produce extracts with moderate and strong activity against some microbes

No.	Strain	Production medium	Antimicrobial activity
1	C194911 (*Streptomyces* sp.)	5294	**Gram-negative bacteria** *Escherichia coli* (moderate) *Escherichia coli* TolC (moderate) *Chromobacterium violaceum* (strong) **Gram-positive bacteria** *Bacillus subtilis* (strong) *Staphylococcus aureus* (strong) *Micrococcus luteus* (strong) **Fungi** *Mucor hiemalis* (strong) *Pichia anomala* (strong) *Candida albicans* (strong)
2	195003R (*Streptomyces* sp.)	5294	**Gram-negative bacteria** *Escherichia coli* TolC (moderate) *Chromobacterium violaceum* (moderate) **Gram-positive bacteria** *Bacillus subtilis* (strong) *Staphylococcus aureus* (strong) *Micrococcus luteus* (strong) *Mycobacterium smegmatis* (strong) **Fungi** *Mucor hiemalis* (moderate)
3	1951016 (*Streptomyces* sp.)	5294	**Gram-negative bacteria** *Escherichia coli* TolC (moderate) **Gram-positive bacteria** *Micrococcus luteus* (moderate)

			Mycobacterium smegmatis (moderate)
			Fungi
			Candida albicans (moderate)
4	196512CR (*Streptomyces* sp.)	5254	**Gram-negative bacteria** *Chromobacterium violaceum* (moderate) **Gram-positive bacteria** *Bacillus subtilis* (strong) *Staphylococcus aureus* (strong) *Micrococcus luteus* (strong) *Mycobacterium smegmatis* (moderate)
			Fungi
			Mucor hiemalis (moderate)
			Pichia anomala (moderate)
5	C196921 (*Streptomyces* sp.)	5254	**Gram-negative bacteria** *Escherichia coli* TolC (moderate) **Gram-positive bacteria** *Bacillus subtilis* (moderate) *Staphylococcus aureus* (moderate)
			Fungi
			Candida albicans (moderate)
6	4421 (*Nocardiopsis* sp.)	5254+SW	**Gram-negative bacteria** *Escherichia coli* TolC (moderate) **Gram-positive bacteria** *Staphylococcus aureus* (moderate) *Mycobacterium smegmatis* (moderate) *Bacillus subtilis* (strong) *Micrococcus luteus* (strong)
7	4423 (*Nocardiopsis* sp.)	5294+SW	**Gram-negative bacteria** *Escherichia coli* TolC (moderate) **Gram-positive bacteria** *Staphylococcus aureus* (moderate) *Mycobacterium smegmatis* (moderate) *Bacillus subtilis* (strong) *Micrococcus luteus* (strong)
8	4431 (*Nocardiopsis* sp.)	5254	**Gram-negative bacteria** *Escherichia coli* TolC (moderate) **Gram-positive bacteria** *Micrococcus luteus* (moderate)
9	180822CR (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Bacillus subtilis* (strong) *Staphylococcus aureus* (strong)
10	195232CR (*Couchioplanes* sp.)	5294	**Gram-negative bacteria** *Escherichia coli* TolC (moderate) **Gram-positive bacteria** *Staphylococcus aureus* (moderate)

			Mycobacterium smegmatis (moderate)
11	194938CR (*Kibdelosporangium* sp.)	5254	**Gram-negative bacteria** *Escherichia coli* TolC (moderate) **Gram-positive bacteria** *Staphylococcus aureus* (moderate)
12	C195212	5254	**Gram-negative bacteria** *Escherichia coli* TolC (moderate) *Chromobacterium violaceum* (moderate) **Gram-positive bacteria** *Bacillus subtilis* (strong) *Micrococcus luteus* (moderate)
13	SHP 6-3 (*Streptomyces* sp.)	5294	**Gram-negative bacteria** *Escherichia coli* TolC (moderate) **Gram-positive bacteria** *Micrococcus luteus* (moderate)
14	190224 (*Streptomyces* sp.)	5254	**Gram-positive bacteria** *Micrococcus luteus* (moderate) **Fungi** *Mucor hiemalis* (moderate) *Pichia anomala* (moderate)
15	190235 (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Staphylococcus aureus* (strong) *Bacillus subtilis* (strong) *Micrococcus luteus* (strong) **Fungi** *Mucor hiemalis* (strong) *Pichia anomala* (strong) *Candida albicans* (strong)
16	195105 (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Bacillus subtilis* (strong) *Staphylococcus aureus* (moderate) *Micrococcus luteus* (moderate) *Mycobacterium smegmatis* (moderate) **Fungi** *Mucor hiemalis* (moderate)
17	195107 (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Bacillus subtilis* (strong) *Staphylococcus aureus* (strong) *Micrococcus luteus* (moderate) *Mycobacterium smegmatis* (moderate) **Fungi** *Mucor hiemalis* (moderate)
18	195334CR (*Amycolatopsis* sp.)	5294	**Gram-positive bacteria** *Staphylococcus aureus* (moderate) *Mycobacterium smegmatis* (moderate)

			Fungi *Mucor hiemalis* (moderate)
19	195337CR (*Streptomyces* sp.)	5254	**Gram-positive bacteria** *Staphylococcus aureus* (strong) **Fungi** *Mucor hiemalis* (moderate)
20	196526CR (*Amycolatopsis* sp.)	5294	**Gram-positive bacteria** *Bacillus subtilis* (moderate) *Staphylococcus aureus* (moderate) **Fungi** *Mucor hiemalis* (moderate) **Candida albicans (moderate)**
21	197019CR (*Streptomyces* sp.)	5254	**Gram-positive bacteria** *Staphylococcus aureus* (moderate) **Fungi** *Mucor hiemalis* (strong)
22	C194922A (*Streptomyces* sp.)	5254	**Gram-positive bacteria** *Bacillus subtilis* (moderate) *Micrococcus luteus* (moderate) **Fungi** *Mucor hiemalis* (moderate)
23	C1950A (*Saccharothrix* sp.)	5294	**Gram-positive bacteria** *Bacillus subtilis* (moderate) *Staphylococcus aureus* (moderate) *Micrococcus luteus* (moderate) **Fungi** *Mucor hiemalis* (moderate)
24	9BLSSO (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Staphylococcus aureus* (moderate) **Fungi** *Mucor hiemalis* (moderate)
25	SHP 1-4 (*Streptomyces* sp.)	5254	**Gram-positive bacteria** *Bacillus subtilis* (moderate) **Fungi** *Pichia anomala* (moderate)
26	SHP 6-6 (*Streptomyces* sp.)	5254	**Gram-positive bacteria** *Bacillus subtilis* (moderate) **Fungi** *Pichia anomala* (moderate)
27	4422 (*Nocardiopsis* sp.)	5294+SW	**Gram-positive bacteria** *Staphylococcus aureus* (moderate) *Bacillus subtilis* (strong) *Micrococcus luteus* (strong)
28	SHP 1-2 (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Bacillus subtilis* (strong) *Staphylococcus aureus* (moderate)

			Micrococcus luteus (strong)
29	4435 (*Nocardiopsis* sp.)	5294+SW	**Gram-positive bacteria** *Micrococcus luteus* (moderate) *Bacillus subtilis* (strong)
30	190231 (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Bacillus subtilis* (moderate) *Micrococcus luteus* (moderate)
31	190234 (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Micrococcus luteus* (moderate)
32	180824CR (*Amycolatopsis* sp.)	5294	**Gram-positive bacteria** *Bacillus subtilis* (moderate)
33	194823CR (*Streptomyces* sp.)	5254	**Gram-positive bacteria** *Bacillus subtilis* (moderate) *Staphylococcus aureus* (moderate)
34	194921CR	5294	**Gram-positive bacteria** *Staphylococcus aureus* (strong)
35	195211CR	5294	**Gram-positive bacteria** *Staphylococcus aureus* (strong)
36	195213BCR (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Staphylococcus aureus* (moderate)
37	195231CR	5294	**Gram-positive bacteria** *Staphylococcus aureus* (moderate)
38	198335CR (*Streptomyces* sp.)	5254	**Gram-positive bacteria** *Bacillus subtilis* (strong)
39	SHP 2-5 (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Bacillus subtilis* (strong) *Micrococcus luteus* (strong)
40	SHP 6-4 (*Streptomyces* sp.)	5294	**Gram-positive bacteria** *Bacillus subtilis* (moderate) *Staphylococcus aureus* (moderate)
41	SHP 7-3 (*Streptomyces* sp.)	5254	**Gram-positive bacteria** *Bacillus subtilis* (strong) *Micrococcus luteus* (strong)
42	190232 (*Streptomyces* sp.)	5294	**Fungi** *Mucor hiemalis* (moderate)
43	190233 (*Streptomyces* sp.)	5294	**Fungi** *Mucor hiemalis* (moderate)
44	194601 (*Streptomyces* sp.)	5294	**Fungi** *Mucor hiemalis* (moderate) *Pichia anomala* (moderate)
45	190224CR (*Streptomyces* sp.)	5254	**Fungi** *Mucor hiemalis* (moderate)
46	195227GnCR (*Streptomyces* sp.)	5254	**Fungi** *Mucor hiemalis* (moderate)
47	196932CR (*Streptomyces* sp.)	5254	**Fungi** *Mucor hiemalis* (moderate)

48	198332CR (*Streptomyces* sp.)	5254	**Fungi** *Mucor hiemalis* (strong) **Candida albicans (strong)**
49	4-5BKBSO (*Pseudonocardia* sp.)	5294	**Fungi** *Mucor hiemalis* (moderate)
50	C190221 (Kitasatospora sp.)	5294	**Fungi** *Pichia anomala* (moderate) *Candida albicans* (moderate)
51	C195321A (*Streptomyces* sp.)	5294	**Fungi** *Mucor hiemalis* (moderate) *Pichia anomala* (moderate)
52	DHE 2-1 (*Kitasatospora* sp.)	5254	**Fungi** *Mucor hiemalis* (strong) *Pichia anomala* (moderate)
53	SHP 1-6 (*Streptomyces* sp.)	5254	**Fungi** *Pichia anomala* (moderate)
54	SHP 2-2 (*Streptomyces* sp.)	5254	**Fungi** *Pichia anomala* (moderate)
55	SHP 2-4 (*Streptomyces* sp.)	5254	**Fungi** *Pichia anomala* (moderate)
56	SHP 6-5 (*Streptomyces* sp.)	5254	**Fungi** *Mucor hiemalis* (moderate) *Pichia anomala* (moderate)
57	SHP 7-1 (*Streptomyces* sp.)	5254	**Fungi** *Mucor hiemalis* (moderate)
58	SHP 7-5 (*Streptomyces* sp.)	5254	**Fungi** *Mucor hiemalis* (moderate) *Pichia anomala* (moderate)

Table S 4. List of nontoxic extracts having antiviral activity with moderate and strong level against HCV

No.	Strain	Extract	Antiviral activity level
1	DHE 2-1	DHE 2-1_5254	Very Strong
2	DHE 2-1	DHE 2-1_5294	Very Strong
3	MAE 1-11	MAE 1-11_5294	Very Strong
4	MAE 1-11	MAE 1-11_SYP	Very Strong
5	SHP 1-4	SHP 1-4_5294	Very Strong
6	SHP 2-2	SHP 2-2_5294	Very Strong
7	SHP 6-6	SHP 6-6_5294	Very Strong
8	190231	190231_5294	Very Strong
9	C190221	C190221_5254	Very Strong
10	C194911	C194911_5294	Very Strong

11	C195321A	C195321A_5254	Very Strong
12	C195321A	C195321A_5294	Very Strong
13	C196921	C19692 _5254	Very Strong
14	4421	4421_5254	Strong
15	4421	4421_5254+SW	Strong
16	4423	4423_5254	Strong
17	4423	4423_5254+SW	Strong
18	4435	4435_5254+SW	Strong
19	5931	5931_5254+SW	Strong
20	5931	5931_5294+SW	Strong
21	SHP 1-5	SHP 1-5_5294	Strong
22	SHP 1-6	SHP 1-6_5294	Strong
23	SHP 6-2	SHP 6-2_5254	Strong
24	SHP 6-2	SHP 6-2_5294	Strong
25	SHP 7-5	SHP 7-5_5294	Strong
26	190401	190401_5254	Strong
27	C194911	C194911_5254	Strong
28	C194922A	C194922A _5254	Strong
29	4422	4422_5254	Moderate
30	4422	4422_5254+SW	Moderate
31	4423	4423_5294	Moderate
32	4431	4431_5254	Moderate
33	4431	4431_5294	Moderate
34	4431	4431_5254+SW	Moderate
35	4433	4433_5254+SW	Moderate
36	4435	4435_5294+SW	Moderate
37	5931	5931_5254	Moderate
38	BLH 12-3	BLH 12-3_5254	Moderate
39	BLH 12-3	BLH 12-3_5294	Moderate
40	DHE 9-4	DHE 9-4_5254	Moderate
41	DHE 9-4	DHE 9-4_5294	Moderate
42	SHP 1-6	SHP 1-6_5254	Moderate
43	SHP 6-5	SHP 6-5_5294	Moderate
44	C195311	C195311_5254	Moderate
45	C196921	C196921_5294	Moderate
46	C194922A	C194922A _5294	Moderate

Table S 5. Phenotypic comparison between *Streptomyces* sp. SHP 1-2, *Streptomyces fumigatiscleroticus* DSM 43154 T, *Streptomyces malachitofuscus* DSM 40332T, *Streptomyces viridochromogenes* DSM 40110 T, and *Streptomyces misionensis* DSM 40306.

Characteristic	1	2	3	4	5
ISP 2 - growth	Good	Good	Good	Good	Sparse
ISP 2 - colony color	Olive brown, ochre yellow	Yellow	Ochre brown	Green	Green brown
ISP 2 - aerial mycelium	Traffic grey B, signal white	Sparse	Grey	Blue grey	None
ISP 2 - soluble pigment	None	None	None	None	None
ISP 3 - growth	Good	Good	Good	Good	Good
ISP 3 - colony color	Ivory	Yellow/ Brown	Yellow	Green	Beige grey
ISP 3 - aerial mycelium	Telegrey 2, signal white	Sparse	White	Blue grey	Beige grey
ISP 3 - soluble pigment	None	None	None	Green	None
ISP 4 - growth	Moderate	Good	Good	Good	Sparse
ISP 4 - colony color	Ivory	Colorless	Colorless	Green	Olive brown
ISP 4 - aerial mycelium	Telegrey 2, signal white	White	Sparse, white	Blue grey	None
ISP 4 - soluble pigment	None	None	None	None	None
ISP 5 - growth	Good	Good	Good	Good	Good
ISP 5 - colony color	Ivory, light ivory	Red brown	Yellow	Green	Beige grey
ISP 5 - aerial mycelium	Telegrey 2, signal white	White	White	Blue grey	Signal white
ISP 5 - soluble pigment	None	None	None	Brown	None
ISP 6 - growth	Good	Good	Good	Good	Sparse
ISP 6 - colony color	Maize yellow	Colorless	Brown	Green	Beige grey
ISP 6 - aerial mycelium	Signal white	None	None	Blue grey	None
ISP 6 - soluble pigment	None	None	Brown	Brown	None
ISP 7 - growth	Good	Good	Good	Good	Sparse
ISP 7 - colony color	Ivory light, ivory	Red brown	Black	Green	Beige grey
ISP 7 - aerial mycelium	Traffic grey A, signal white	Grey	White	Blue grey	Dusty grey
ISP 7 - soluble pigment	None	Red	Black	None	None
Use of carbohydrate					
Glucose	+	-	+	+	+
Arabinose	-	+	+	+	(+)
Sucrose	-	-	+	-	-
Xylose	-	-	-	+	++
Inositol	-	-	+	+	(+)
Mannitol	-	+	(+)	+	(+)
Fructose	-	+	+	+	(+)
Rhamnose	-	+	+	-	-

Raffinose	-	+	-	-	-
Cellulose	-	-	-	-	-
API ZYM					
Phosphatase alcaline	++	++	++	++	++
Esterase (C4)	(+)	+	(+)	+	+
Esterase Lipase (C8)	(+)	(+)	(+)	+	++
Lipase (C14)	(+)	-	-	-	(+)
Leucin arylamidase	++	++	++	++	++
Valine arylamidase	(+)	++	+	+	++
Cystine arylamidase	(+)	+	+	+	+
Trypsin	-	(+)	+	+	-
Chymotrypsin	-	(+)	+	(+)	(+)
Phosphatase acid	(+)	++	++	++	++
Naphtol-AS-BI-phosphohydrolase	(+)	++	++	++	++
α-Galactosidase	-	(+)	-	-	+
β-Galactosidase	(+)	++	+	++	++
β-Glucuronidase	-	-	-	-	-
α-Glucosidase	++	+	++	-	++
β-Glucosidase	(+)	++	(+)	++	-
N-acetyl-beta-glucoseamidase	++	-	+	++	++
α-Mannosidase	-	++	++	++	++
α-Fucosidase	-	-	-	-	-
API Coryne					
Nitrate reduction	-	+	-	-	-
Pyraziamidase	-	-	-	-	-
Pyrrolidonyl arylamidase	-	-	-	-	+
Alkaline phosphatase	+	+	+	+	+
β-Glucuronidase	-	-	-	-	-
β-Galactosidase	-	+	-	-	+
α-Glucosidase	+	+	+	-	+
N-acetyl -beta glucoseamidase	+	+	-	+	+
Esculin (beta glucosidase)	+	+	-	-	+
Urease	-	-	-	-	-
Gelatine(hydrolysis)	+	+	+	-	+
Glucose fermentation	-	-	-	-	-
Ribose fermentation	-	-	-	-	-
Xylose fermentation	-	-	-	-	-
Mannitol fermentation	-	-	-	-	-
Maltose fermentation	-	-	-	-	-
Lactose fermentation	-	-	-	-	-
Sucrose fermentation	-	-	-	-	-
Glycogen fermentation	-	-	-	-	-

++ more positive result; + positive result; - negative result; (+) weakly positive result; 1: Strain SHP 1-2; *2: Streptomyces fumigatiscleroticus* DSM 43154 T; 3: *Streptomyces malachitofuscus* DSM 40332; 4: *Streptomyces viridochromogenes* DSM 40110; 5: *Streptomyces misionensis* DSM 40306.

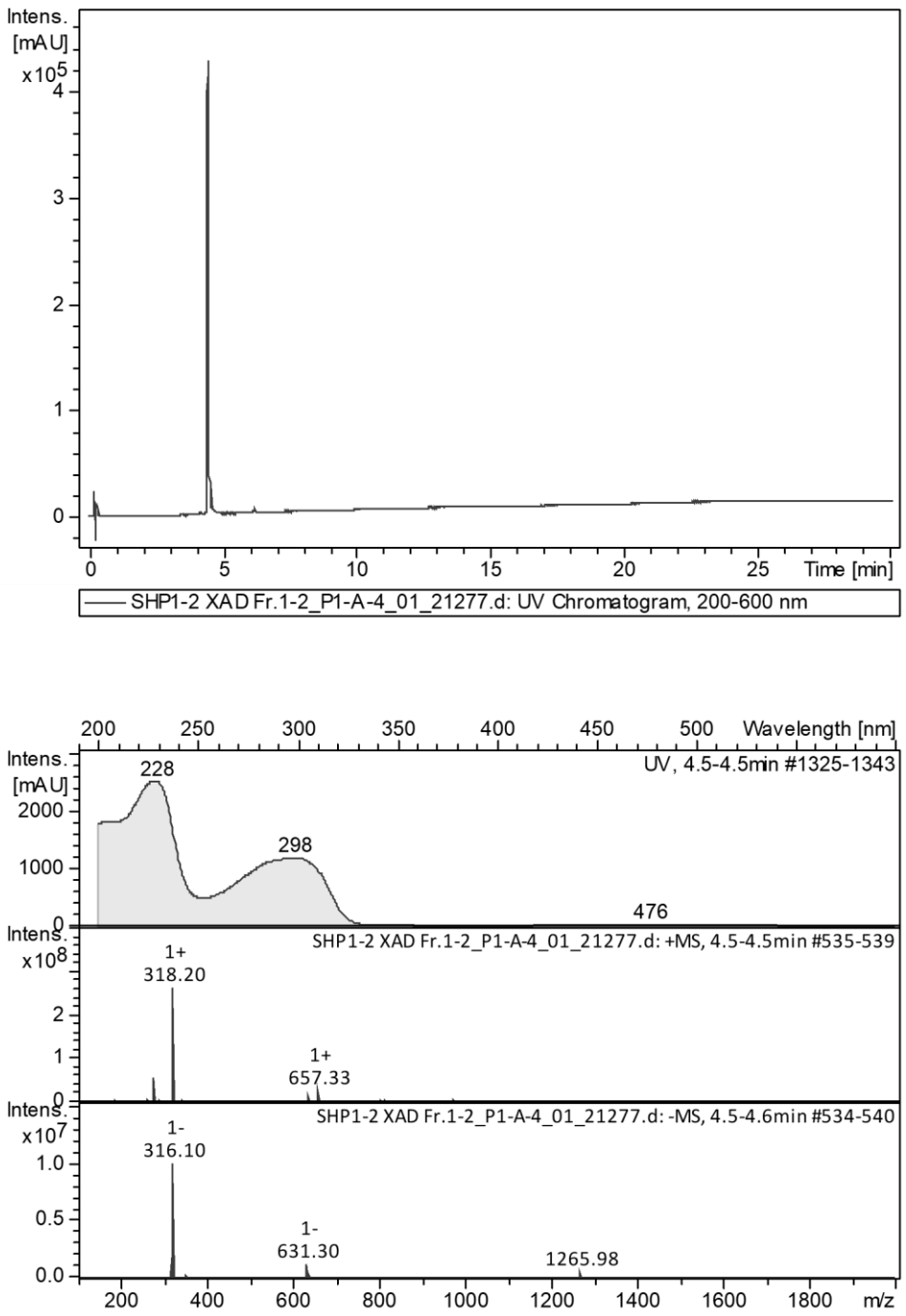

Figure S 1. Chromatogram and spectra of Indolactam variant 1.

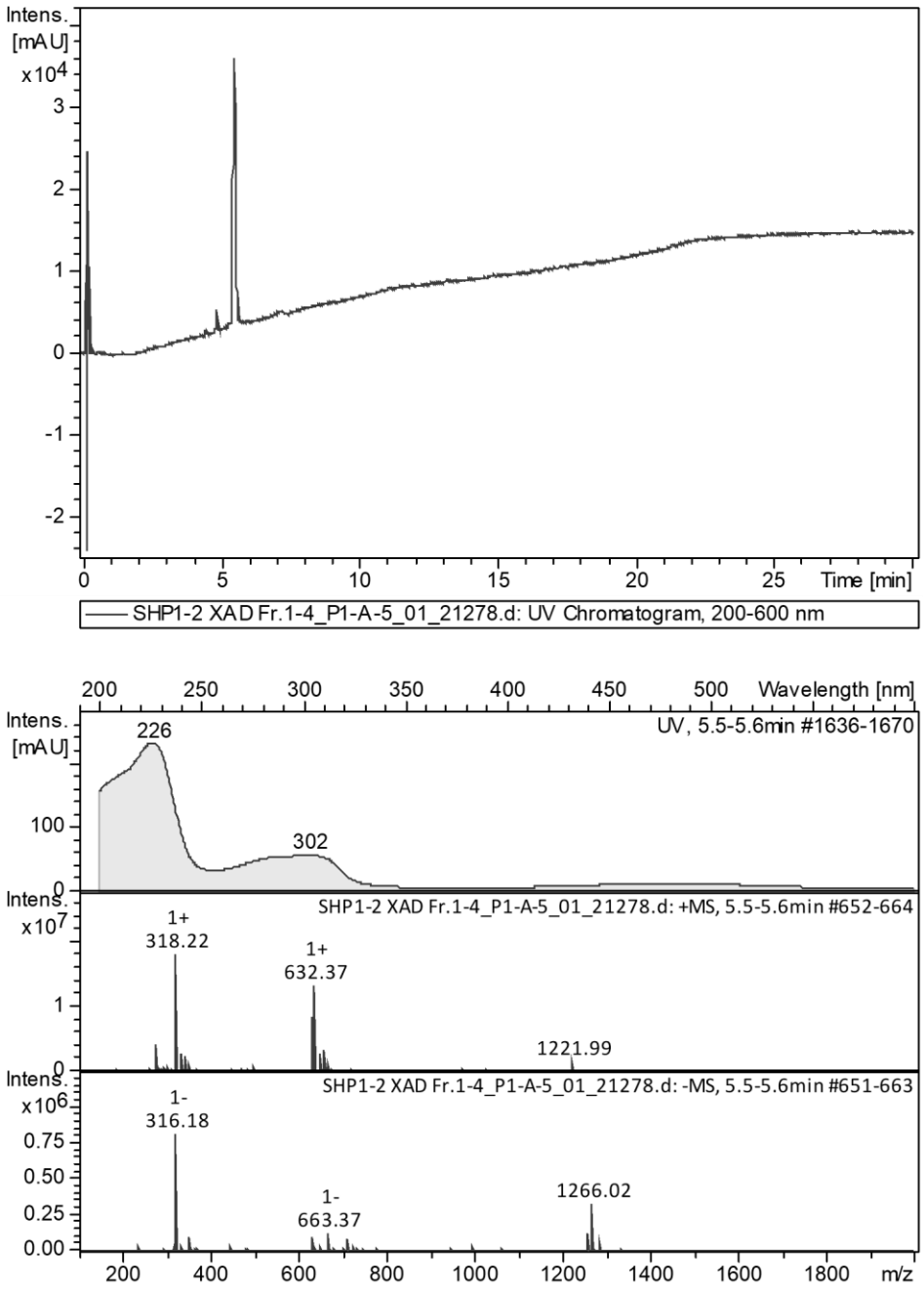

Figure S 2. Chromatogram and spectra of Indolactam variant 2.

Table S 6. NMR data of indolactam variant 1

No	No. Atom	C Shift	XHn	H Shift	H Multiplicity	COSY	N/ROESY	H to C HMBC	C to H HMBC	TOCSY	H Mark
1	19	16.852	CH3	0.965	d (6.45)	2.57	2.57, 2.99, 3.47, 4.68	2.57, 2.99, 3.47, 4.68	37.25, 64.70, 68.56	2.57, 2.99, 3.47, 4.68	
2	20	33.947	CH3	2.897	s		2.57, 2.99, 3.47, 4.15, 6.49	4.68	68.56, 106.58, 148.43		
3	10	35.514	CH2	3.082	dd (17.21, 3.76)		3.47, 3.67, 4.68, 7.03	3.47, 7.03	65.76, 119.71	7.03	"
4	10	35.514	CH2	3.107	m	7.03	3.67, 4.15, 4.68, 6.35, 7.03	3.47, 7.03	57.11	3.47, 7.03	'
5	17	37.246	CH	2.567	dqdt (10.10, 6.45, 6.45, 6.45, 4.00, 3.23, 3.23)	0.96, 2.99, 4.68	0.96, 2.90, 2.99, 3.47, 3.47, 4.68	0.96, 4.68	16.85, 68.56, 172.95	0.96, 2.99, 3.47, 3.47, 4.68	
6	11	57.112	CH	4.147	dqd (9.00, 4.40, 4.40, 4.30, 3.87)	3.09, 3.47, 3.67	2.90, 3.11, 3.47, 3.67, 4.68, 6.35	3.11, 3.47		3.09, 3.47, 3.67, 6.35	
7	18	64.701	CH2	3.470	dd (10.54, 3.00)	2.99	0.96, 2.57, 2.90, 6.49	0.96, 4.68	16.85, 68.56	0.96, 2.57, 4.68	"
8	18	64.701	CH2	2.987	dd (10.54, 4.20)	2.57, 3.47	0.96, 2.57, 2.90, 3.47, 4.68, 6.49	0.96, 4.68	16.85	0.96, 2.57, 4.68	'
9	16	65.758	CH2	3.475	dd (11.00, 9.00)	3.67, 4.15	2.57, 2.99, 3.08, 3.67, 4.15, 4.68, 6.35	3.08	35.51, 57.11	2.57, 3.09, 3.11, 3.67, 4.15, 6.35	
10	16	65.758	CH2	3.666	dd (11.00, 4.41)	3.47, 4.15	3.08, 3.11, 3.47, 4.15, 6.35	3.08		3.09, 3.47, 4.15, 6.35	
11	14	68.556	CH	4.682	d (10.11)	2.57	0.96, 2.57, 2.99, 3.08, 3.11, 3.47, 4.15	0.96, 2.57, 2.90, 3.47	16.85, 33.95, 37.25, 64.70, 148.43, 172.95	0.96, 2.57, 2.99, 3.47	
12	8	106.061	CH	6.934	d (8.00)		10.05	6.49, 106.58	106.58, 119.71	6.49	
13	6	106.581	CH	6.488	d (7.53)	6.97	2.90, 2.99, 3.47	2.90, 6.93, 6.97, 106.06	106.06, 115.31, 119.71	6.93	
14	3	115.314	C					3.09, 6.49, 7.03			

15	4	119.715	C					3.08, 6.49, 6.93, 6.97, 7.03			
16		123.128	C								
17	2	123.492	CH	7.035	s	3.11	3.08, 3.11, 10.05	3.09	35.51, 115.31, 119.71	3.08, 3.11, 10.05	
18	9	141.131	C					6.97			
19	5	148.435	C					2.90, 4.68, 6.97			
20	13	172.953	C					2.57, 4.68			
21				3.093	m	4.15			115.31, 123.49	3.47, 3.67, 4.15	
22	12		NH	6.353	br s		3.11, 3.47, 3.67, 4.15			3.47, 3.67, 4.15	
23	1		NH	10.046	br s		6.93, 7.03			7.03	
24	7		CH	6.973	dd (8.00, 7.53)	6.49			106.58, 119.71, 141.13, 148.43		

Table S 7. NMR data of indolactam variant 2

No	Atom No.	C Shift	XHn	H Shift	H Multiplicity	COSY	N/ROESY	H to C HMBC	C to H HMBC	TOCSY	H Mark
1	19	15.570	CH3	0.676	d (7.10)	2.58	2.58, 2.88, 3.44, 3.58, 4.92, 6.44	2.58, 3.44, 3.58, 4.92	37.45, 67.20, 173.96	2.58, 3.44, 3.58, 4.92	
2	20	33.720	CH3	2.879	s		0.68, 2.58, 4.15, 4.92, 6.44	4.92	67.20, 106.68, 149.15		
3	10	35.072	CH2	3.199	br dd (17.21, 4.09)	3.01, 4.15, 7.00	3.01, 3.66, 4.15, 4.92, 7.00	3.47, 3.66, 7.00	57.20, 65.73, 115.68, 123.09	3.01, 3.47, 3.66, 4.15, 7.00	'
4	10	35.072	CH2	3.013	dd (17.21, 3.76)	3.20, 4.15	3.20, 3.47, 3.66, 4.15, 7.00	3.47, 3.66, 7.00	57.20, 65.73, 115.68, 119.62, 123.09	3.20, 3.47, 3.66, 4.15, 7.00	"
5	17	37.446	CH	2.579	dqdd (10.40, 7.10, 7.10, 7.10, 3.66, 3.23)	0.68, 3.44, 3.58, 4.92	0.68, 2.88, 3.44, 3.58, 4.15, 4.92	0.68, 3.44, 3.58, 4.92	15.57, 67.20, 173.96	0.68, 3.44, 3.58, 4.92	

6	11	57.199	CH	4.150	ddddd (9.40, 4.52, 4.40, 4.09, 3.76)	3.01, 3.20, 3.47, 3.66	2.58, 2.88, 3.01, 3.20, 3.47, 3.66, 4.92, 6.37	3.01, 3.20, 3.47	65.73, 115.68	3.01, 3.20, 3.47, 3.66	
7	16	65.732	CH2	3.661	dd (11.19, 4.52)	3.47, 4.15	3.01, 3.20, 3.47, 3.58, 4.15, 6.37	3.01, 3.20, 3.38, 4.15	35.07	3.01, 3.20, 3.47, 4.15	
8	16	65.732	CH2	3.467	dd (11.00, 9.25)	3.66, 4.15	3.01, 3.58, 3.66, 4.15, 6.37	3.01, 3.20, 3.38, 4.15	35.07, 57.20	3.01, 3.20, 3.66, 4.15	
9	18	66.469	CH2	3.581	dd (10.54, 3.66)	2.58, 3.44	0.68, 2.58, 3.44, 3.47, 3.66, 4.92	4.92	15.57, 37.45	0.68, 2.58, 3.44, 4.92	
10	18	66.469	CH2	3.442	dd (10.54, 3.23)	2.58, 3.58	0.68, 2.58, 3.58, 4.92	4.92	15.57, 37.45, 67.20	0.68, 2.58, 3.58, 4.92	
11	14	67.205	CH	4.919	d (10.40)	2.58	0.68, 2.58, 2.88, 3.20, 3.44, 3.58, 4.15	0.68, 2.58, 2.88, 3.44, 6.44	15.57, 33.72, 37.45, 66.47, 149.15, 173.96	0.68, 2.58, 3.44, 3.58	
12	8	105.567	CH	6.897	dt (8.00, 0.80, 0.80)		9.98	6.44	106.68, 119.62, 149.15	6.44	
13	6	106.676	CH	6.440	d (7.53)	6.95	0.68, 2.88	2.88, 6.90, 6.95	67.20, 105.57, 115.68, 119.62, 149.15	6.90	
14	3	115.678	C					3.01, 3.20, 4.15, 6.44, 7.00			
15	4	119.620	C					3.01, 6.44, 6.90, 7.00			
16	2	123.094	CH	7.003	d (1.29)	3.20	3.01, 3.20, 9.98	3.01, 3.20	35.07, 115.68, 119.62, 141.24	3.01, 3.20	
17	7	123.406	CH	6.948	dd (8.00, 7.53)	6.44			106.68, 141.24, 149.15		
18	9	141.235	C					6.95, 7.00			
19	5	149.154	C					2.88, 4.92, 6.44, 6.90, 6.95			
20	13	173.958	C					0.68, 2.58, 4.92			
21	1		NH	9.981	br s		6.90, 7.00				
24	12		NH	6.369	br s		3.47, 3.66, 4.15				

Table S 8. NMR data of nitrososaxin C

Nitrososaxin C[a] ($CDCl_3$)		**6526-61-63 ($CDCl_3$)**	
^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR
0.86 (6H, d, 6.85 Hz)	22.63	0.87 (6H, d, 6.45)	22.64
1.13-1.32 (18H, m)	26.12	1.14-1.27 (18H, m)	27.10
1.51 (1H, m)	26.59	1.52 (1H, dt, 13.28 Hz, 6.59 Hz)	27.11
1.94 (2H, q, 7.26 Hz)	27.38	1.71-1.81 (2H, br, s)	27.41
4.13 (2H, t, 7.26 Hz)	27.95		27.96
11.52 (1H, s)	28.81		29.11
	29.27		29.29
	29.45		29.46
	29.55		29.57
	29.64		29.64
	29.89		29.93
	39.04		39.04
	61.38		65.12[b]

[a]Nishio *et al*., 1993[226]; [b]determined in HSQC-DEPT